Steroid signalling in the human ovarian surface epithelium wound healing

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I would like to dedicate this thesis to my parents, Nikos and Maria, my siblings, Petros, Antreas and Chrysoula and my little niece, Dimitra

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world." **Louis Pasteur**

DECLARATION

I hereby declare that I am the author of this thesis and that I did all the work described herein, unless otherwise specified. All sources have been acknowledged by the means of references.

Georgia Papacleovoulou

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List of Abbreviations

A

aa: amino acids

A-II: angiotensin-II

ABC: avidin-biotin complex

ACTH: adrenocorticotropin hormone

AJCC: American Joint Committee of Cancer

AKR: aldoketoreductase

ANOVA: analysis of variance

AP-2: activator protein-2

AR: androgen receptor

ARA-70: androgen receptor-associated protein 70

Asn: asparagine

Asp: aspartic acid

ATF-2: activating transcription factor-2

B

BAY11: BAY117082

BMP: bone morphogenetic protein

bp: base pair

BSA: bovine albumin serum

C

cAMP: 3'-5'-cyclic adenosine monophosphate

CD: common docking site

CG: chorionic gonadotrophin

CK: cytokeratin

COX-2: cyclooxygenase 2

Cys: cysteine

D

DAB: diaminobenzidine

Dapi: 4,6-diamidino-2-phenylindole

DCS: donor calf serum

DiagLapar: diagnostic laparoscopy

Dlx-3: distal-less 3

DHEA-S: dehydroepiandrosterone sulfate

DPBS: dulbecco's phosphate buffer saline

E

ECL: enhanced chemiluminescence

ECM: extracellular matrix

EGF: epidermal growth factor

EOC: epithelial ovarian cancer

ER: oestrogen receptor

ERK: extracellular signal-regulated kinase

F

FBS: foetal bovine serum

FIGO: International Federation of Obstetrics and Gynaecology

FGF: fibroblast growth factor

FH-OSE: family history OSE

FRET: fluorescence resonance energy transfer

FSH: follicle stimulating hormone

FW: Forward

G

G: grade

Glu: glutamine

Gly: glycine

GM-CSF: granulocyte macrophage-colony stimulating factor

GnRH: gonadotrophin releasing hormone

GPCR: G-protein couple receptor

GR: glucocorticoid receptor

H

H+E: haematoxylin and eosin

HMB: heavy menstruation bleeding

His: histidine

hOSE: human ovarian surface epithelium

HRE: hormone response elements

HRP: horseradish peroxidase

HSD: hydroxysteroid dehydrogenase

I

IARC: International Agency for Research on Cancer

IF: immunofluorescence

IFN-γ: interferon-γ

IGF: insulin growth factor

IHC: immunohistochemistry

IκB: inhibitory κappa B

IKKs: inhibitory κappa B kinases

Ile: isoleucine

IL: interleukin

IL-1R: IL-1 receptor

IL-1R1: IL-1 receptor type 1

IL-1R2: IL-1 receptor type 2

IL-1Ra: interleukin-1 receptor antagonist

IL-1RacP: IL-1R accessory protein

IL-4R: IL-4 receptor

IRAK-1: IL-1-related associated kinase 1

IRS: insulin receptor substrate

J

Jak: Janous-family tyrosine kinases

JNK: Jun N-terminal kinase

K

Km: Michaelis constant

L

LAVH: laparoscopic assisted vaginal hysterectomy

LEF: leflunomide

LH: luteinising hormone

LOX: lysyl oxidase

LREC: Lothian Research Ethical Committee

Lys: lysine

LY94: LY94002

M

MAPK: mitogen activated protein kinase

MAPKK: MAPK kinase

M-CSF: macrophage colony-stimulating factor

MCP-1: monocyte chemotactic protein-1

Met: methionine

MKKs: mitogen-activated protein kinase kinases

MKPs: MAPK

MMPs: matrix metalloproteinases

mTLD: mammalian toloid

N

NADPH: reduced nicotinamide adenine dinucleotide phosphate-oxidase

NFH-OSE: no family history-OSE

NF-κB: nuclear factor-kappa B

NGF: neural growth factor

NGS: normal goat serum

NOS: adenocarcinomas not specified

NTC: non-template control

P

P450arom: P450 aromatase

P450scc: P450 cholesterol side chain cleavage

PAGE: polyacrylamide gel electrophoresis

PARP: poly-(ADP) ribose polymerase

PBST: PBS + 0.05% Tween 20

PCOS: polycystic ovarian syndrome

PCP: procollagen C-terminal protein

PCPE: PCP enhancer

PCR: polymerase chain reaction

PD98: PD98059

PID: pelvic inflammatory disease

PI-3K: phosphatidyl-inositol-3 kinase

PKA: protein kinase A

PKC: protein kinase C

PMA: phorbol-12-myristate-13-acetate

PMN: polymorphonuclear

PR: progesterone receptor

Preg/P5: pregnenolone

Prog/P4: progesterone

Pro: proline

Q

qPCR: quantitative PCR

R

R: receptor

RIE: Royal Infirmary of Edinburgh

RT: Reverse Transcription

RT-ve: RT negative

RV: reverse

S

SDR: short-chain alcohol dehydrogenase/reductase

SDS: sodium dodecyl sulfate

SEM: scanning electron microscopy

S: serine

SAPK/JNK: stress/osmotic associated protein kinase/jun N-terminal kinase

SB20: SB203580

Ser: serine

SF-1: steroidogenic factor-1

SOCS-3: suppressor of the cytokine signalling-3

sqPCR: semi-quantitative PCR

SRC-1: steroid receptor co-activator-1

STAH: sub-total abdominal hysterectomy

StAR: steroid acute regulatory protein

STAT: signal transducers and activators of transcription protein

STS: steroid sulfatase

sem: standard error of the mean

T

TAH: total abdominal hysterectomy

TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy

TAK-1: TGF-β-activated kinase-1

TEF-5: transcription enhancer factor 5

TGF-β1: transforming growth factor-β1

Th-2: T-helper 2

Thr: threonine

TIMPs: tissue inhibitor of matrix metalloproteinases

TIR: toll-like/IL-1 receptor

TLC: thin layer chromatography

TLR: toll-like receptor

Tm: melting temperature

TNF-α: tumour necrosis factor-α

TRAF-6: TNF receptor associated factor-6

Trp: tryptophan

Tyr: tyrosine

U

uPA: urokinase plasminogen activator

UNG: uracil-N-glycosylase

UV: ultraviolet light

V

VEGF: vascular endothelial growth factor

Vmax: maximal velocity

W

W: tryptophan

WHO: World's Health Organisation

Y

Y: tyrosine

Z

ZF: zona fasciculata

ZG: zona glomerulosa

ZR: zona reticularis

Misc

3β-HSD/KSI: 3β-hydroxysteroid dehydrogenase/ketosteroid isomerase

5α-DHT: 5α-dihydrotestosterone

11β-HSD: 11β-hydroxysteroid dehydrogenase

17-βHSD: 17β-hydroxysteroid dehydrogenase

γc: γ common chain

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ABSTRACT OF THESIS

The human ovarian surface epithelium (hOSE) is a cell monolayer that covers the surface of the ovary. Natural events like incessant ovulation, associated reproductive hormone action prior to and post-ovulation, along with the ovulation-associated inflammation, that result in injury and repair of hOSE, are considered to have a role in the development of epithelial ovarian cancer (EOC). Progesterone is apoptotic and anti-inflammatory, whereas androgens appear cytoproliferative for hOSE. Local generation of these steroid hormones is subject to 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. Moreover, action of these hormones is achieved through coupling to their cognate receptors, progesterone (PR) and androgen receptors (AR). The overall aim of this thesis is to elucidate *in vitro* the regulation of progesterone and androgen biosynthesis and downstream signalling during the injury and repair of primary hOSE cells that were collected from pre-menopausal women who underwent surgery for benign gynaecological disorders. Injury was mimicked by treatment of cells with several pro-inflammatory cytokines, whereas repair was mimicked with T-lymphocyte, 'anti-inflammatory' cytokines.

Immunohistochemical studies showed immunodetectable 3β-HSD in the human ovarian cell surface of whole ovary and three-week cultured hOSE cells, establishing 3β-HSD expression *in vivo* and *in vitro*. Cross-reaction of the 3β-HSD antibody with both enzyme isoforms did not allow investigation of isoform expression pattern. However, mRNA transcriptional studies with isoform specific primers and probe sets for semi-quantitative (sq) and quantitative (q) PCR revealed expression of both isoforms in hOSE cells; 3β-HSD1 mRNA was expressed at higher levels relative to 3β-HSD2 mRNA in accordance with the preference of this isoform in peripheral non-steroidogenic tissues.

Of the cytokines tested, only IL-1α and IL-4 affected 3β-HSD expression. IL-1α suppressed 3β-HSD1 mRNA, whereas it up-regulated 3β-HSD2 mRNA as assessed with qPCR, without though affecting total 3β-HSD protein and activity

levels as assessed with western immunoblotting and radiometric activity assays, respectively. IL-1α did not affect AR or PR mRNA levels, suggesting a balance in androgen and progesterone biosynthesis during post-ovulatory wounding. IL-4 massively induced 3β-HSD1 and 3β-HSD2 mRNA and total 3β-HSD protein and activity. It also attenuated AR mRNA and protein, without affecting PR mRNA. Collectively, these data demonstrate that IL-4 sustains progesterone rather than androgen signalling and this may be part of the anti-inflammatory steroid action that protects hOSE from genetic damage. IL-1α effects appear to be mediated by NF-κB signalling pathway. PI-3K and p38 MAPK appeared involved in IL-1α-induced 3β-HSD2. IL-4-induced 3β-HSDs required STAT-6 and PI-3K pathways and also p38 MAPK at the case of 3β-HSD2. IL-4-attenuated AR was reversed by a p38 MAPK inhibitor. These data suggest that steroid signalling by IL-1α and IL-4 involve multiple signalling pathways.

In primary EOC, 3β-HSD1 and 3β-HSD2 transcripts were attenuated relative to hOSE cells, suggestive of an acquired feature of neoplastic transformation. However, both transcripts could be restored after IL-4 treatment, attesting a therapeutic advantage of this cytokine.

In conclusion, we have shown that 3β-HSD is under inflammatory control during ovarian post-ovulatory wound healing of hOSE. IL-1α- and IL-4-mediated 3β-HSD1 and 3β-HSD2 are regulated by multiple signalling pathways. Also, IL-4 was identified as an anti-inflammatory agent in hOSE with putative therapeutic benefit in malignancy.

Chapter 1

General introduction and literature review

1.1 The Female Reproductive Tract

The female reproductive tract is encompassed by the vagina, uterus and the ovary. The vagina is attached to the uterus through the cervix, whilst the ovaries are linked to the uterus through the oviduct or fallopian tubes (Fig. 1.1). The ovaries and the uterus are the most active organs of the female genital tract, since the ovary is the source of the release of oocyte in the oviduct, where it can be fertilised, and pass to the uterus, the site of blastocyst implantation.

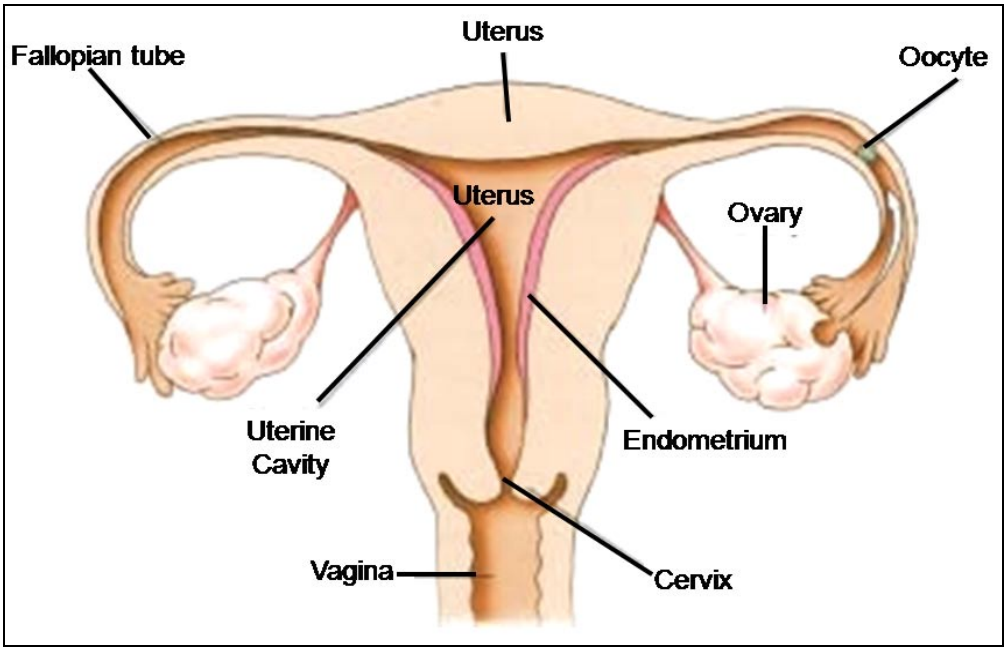

Figure 1.1: The female reproductive tract. Major components of female reproductive tract. Cervix links uterus with vagina and ovary is attached to uterus through the fallopian tube. Downloaded and moderately modified after http://www.humanillnesses.com/original/images/hdc_0001_0002_0_img0170.jpg (2008).

The functions of the ovary and uterus are subject to endocrine, paracrine and intracrine signals that alter periodically at different stages of the menstrual cycle. Orchestration of ovarian and uterine function is a pre-requisite for the homeostasis of the female reproductive system. Any deficiency in hormonal coordination that results in dysfunction of the female reproductive organs could give rise to reproductive disorders such as infertility and more importantly cancer.

This thesis is focused on the biology and specifically the intracrinology of the human ovarian surface epithelium (hOSE) of the pre-menopausal ovary. This cellular compartment is essentially the cover of the ovary and its study has become important when it was realised that it was the origin of more than 85% of ovarian carcinomas (Auersperg *et al.* 2001, Runnebaum & Stickeler 2001). Therefore, studies that are described herein aim at a better understanding of the physiologic function of hOSE that could potentially improve prognosis rates for ovarian disorders with great interest in ovarian cancer.

In order to understand further the mechanisms which physiologically regulate hOSE along with the mechanisms that can lead to malignancy, an overview of the biology of the ovary as a whole organ is essential. As such, this chapter is focused on the biology and endocrinology of the different ovarian compartments, including the human ovarian surface epithelium.

1.2 The Human Ovary

1.2.1 Anatomy and embryology of the human ovary

The adult ovaries are paired organs found in the abdominal cavity located at either side of the uterus. They are ovoid in shape with variable size and weight, depending on the content of their follicular derivatives. The adult ovary consists of follicles of different developmental stages, corpora lutea and corpora albicantia that are scattered among three ill-defined zones of stroma: the outer cortex, the inner medulla and the hilus. The external layer of the outer cortex is commonly referred to

as the tunica albuginea; the latter has a fibrotic appearance, consisting of five to seven layers of fibroblast and collagen (Espey 1994). All the structures that organise the ovary are surrounded by a single epithelial cell layer, the ovarian surface epithelium (OSE), that is separated from the inner ovarian compartments by a well-structured basal lamina (Clement 1994) (Fig. 1.2).

Gonads start to develop on 3rd week of gestation. At this time gonad formation is common between the two sexes. The indifferent gonads arise from proliferation of the coelomic epithelium into the mesenchyme. Mesonephric embryonic cells also invade the mesenchyme. The primordial germ cells migrate from the yolk sac to the urogenital ridge where the mesodermal (in origin) coelomic epithelium starts to proliferate, givimg rise to the surface epithelium of the gonad, while the gonad itself starts to divide. Differentiation of the gonad into the female ovary starts on the 8th week of gestation and is subject to the absence of testicular sex cords due to the lack of *testis determining factor*. On the other hand, the primordial germ cells along with mesonephric cells are joined together and are incorporated to the surface epithelial cells, forming a thick cortex. Proliferating epithelial cortical cells of probable mesonephric origin start then to divide into primitive granulosa cells that surround the germ cells or oocytes, encompassing the primordial follicles that enter meiosis and stop to proliferate until puberty. Primordial follicles are scattered in a fibrous tissue, the stroma, which is probably derived from the mesenchyme. All these structures are kept together and lined by an ovarian surface epithelium that originates from the laterally underlying coelomic epithelium that enwraps the developing female gonad (Chen *et al.* 2003, Okamura *et al.* 2003, Robboy 1994).

1.2.2 Histology of the human ovary

1.2.2.1 The follicle

From birth, primordial follicles stay at the meiotic pro-phase until puberty when they start to develop progressively to follicles or die through atresia. Therefore, whereas at birth, about 400,000 primordial follicles are scattered within the female gonad, this number decreases from puberty until disappearance at the end of menopause. The primordial follicles consist of a layer of squamous quiescent granulosa cells, the pre-granulosa cells that surround the oocyte. When reproductive life starts, the primordial follicles pass into different maturing stages namely primary follicle, pre-antral follicle, antral follicle and pre-ovulatory/Graafian follicle (Fig. 1.2). In each monthly menstrual cycle, only one follicle normally undergoes full maturation and is capable of ovulation and fertilisation, whilst the others become atretic at earlier developmental phases. Early follicular maturation starts at the luteal phase of the menstrual cycle when around three follicles compete for ovulation; only one, however, will be competent to respond to the hormonal events at the follicular phase and can undergo ovulation (Clement 1994).

Primary follicles have approximately the same size as the primordial follicles. Transition to the primary follicle involves differentiation of flat granulosa cells to a more columnar/cuboidal phenotype that are subsequently proliferate to give 5 cell layers, forming the pre-antral follicle. Pre-antral follicles are larger in diameter, they migrate in a more vascularised environment within the medulla where the surrounding stroma is differentiated in the theca interna and externa. Although they become active, they do not enter the reactivation of meiosis yet. Oocyte ribosomal and mRNA synthesis which will give rise to proteins essential for later stages of the oocyte maturation also occurs (Johnson & Everitt 2000a). The zona pellucida is now completely formed to separate the oocyte from the surrounding granulosa cell layers which form gap junctions at the oocyte surface through which oocyte-granulosa cell interactions are achieved (Johnson & Everitt 2000a, Sommersberg *et al.* 2000, van Wezel & Rodgers 1996). In essence, gap junctions are believed to protect the cells

from death signals (Amsterdam *et al.* 2003). Further proliferation of granulosa cells along with secretion of mucopolysaccharide-rich fluid results in the formation of the antral cavity that encompasses the antral follicle. This is also accompanied by an increase in RNA and protein synthesis within the oocyte. The oocyte, in turn, is increased in size and orientates to the one side of the antrum where granulosa cells proliferate to form the cumulus oophorus that encloses the oocyte. At that stage, final maturation of the oocyte occurs, forming the Graafian follicle that is ready to release the fertilisable oocyte (Clement 1994, Fortune 2003, Rodgers *et al.* 2001). Interestingly, at this stage the membrana granulosa is enveloped by a basal lamina and comprises granulosa cells that vary in the levels of gene expression, shape and age, indicating the dynamic role of this cell population during follicular development (Rodgers *et al.* 2001).

1.2.2.2 The corpus luteum

The human corpus luteum is one of the most active steroidogenic tissues of the body. It consists of small and large luteal cells that arise from the theca and granulosa cells of the ovulatory follicle respectively. Unlike small luteal cells that have few mitochondria and lack granules, large luteal cells consist of electron-dense granules and are rich in mitochondria and lipid droplets (Cooke 1988). The physiological relevance of these two cell types (small and large) is unclear, however, studies in animal models have shown that they are differentially regulated and they possess distinct steroidogenic properties (Hawkins *et al.* 1993, Juengel *et al.* 1994). Furthermore, the corpus luteum is rich in fibroblasts, endothelial and immune cells.

1.2.2.3 The human ovarian surface epithelium (hOSE)

As noted earlier, the human ovarian surface epithelium or hOSE is of mesodermal origin, arising from the proliferating coelomic epithelium. hOSE comprises a cell layer that lines the ovary and separates it from other peritoneal epithelial structures (Fig. 1.2). It is distinguished from the underlying cortical stroma by a well-formed basal lamina. Intriguingly, OSE is identical to the extraovarian

mesothelium, because both share the same origin and experience a similar environment (pelvic cavity). However, it can be distinguished from the rest of coelomic epithelium derivatives by the presence of 17β-hydroxysteroid dehydrogenase activity (17β-HSD) (Blaustein 1984) and the absence of CA125 glycoprotein (Kabawat 1983). Notably, the presence of the latter in ovarian neoplasms is reflective of the epithelial-committed phenotype of ovarian carcinomas as opposed to the epithelial-mesenchymal transitions of the ovarian cell surface (Auersperg *et al.* 1984, Kruk *et al.* 1990). This shows that hOSE keeps the features of a pleuripotential coelomic epithelium as opposed to the other derivatives of the same embryonic origin that are more committed to their epithelial phenotype (Auersperg *et al.* 2001).

Scanning electron microscopy (SEM) has revealed that the hOSE is encompassed by a heterogenous cell population ranging from a cuboidal (rounded and columnar) to a squamous (flat) appearance in a well-defined and distinct manner (Gillett *et al.* 1991). It has been reported that squamous cells are present at the apex of the corpus luteum, suggesting that those cells are probably a result of post-ovulatory repair (Gillett *et al.* 1991). Others have noticed those cell-like appearance at papillary outgrowths as well, reflecting probably a pre-neoplastic appearance of OSE (Auersperg *et al.* 1984, Nicosia *et al.* 1984). Moreover, those cells appear to have reduced numbers of microvilli relative to cuboidal-like cells (Gillett *et al.* 1991). However, in general, OSE cells are laterally joined the one to the other with tight gap junctions. Nuclei are quite large and sometimes possess two or three lobes. In squamous flat cells, nuclei are elongated and parallel to the cell surface. Golgi apparatus and lipid droplets within the cytoplasm do not differ to a great extent between the two cell types, whilst the mitochondria are more dense in cuboidal cells (Papadaki & Beilby 1971).

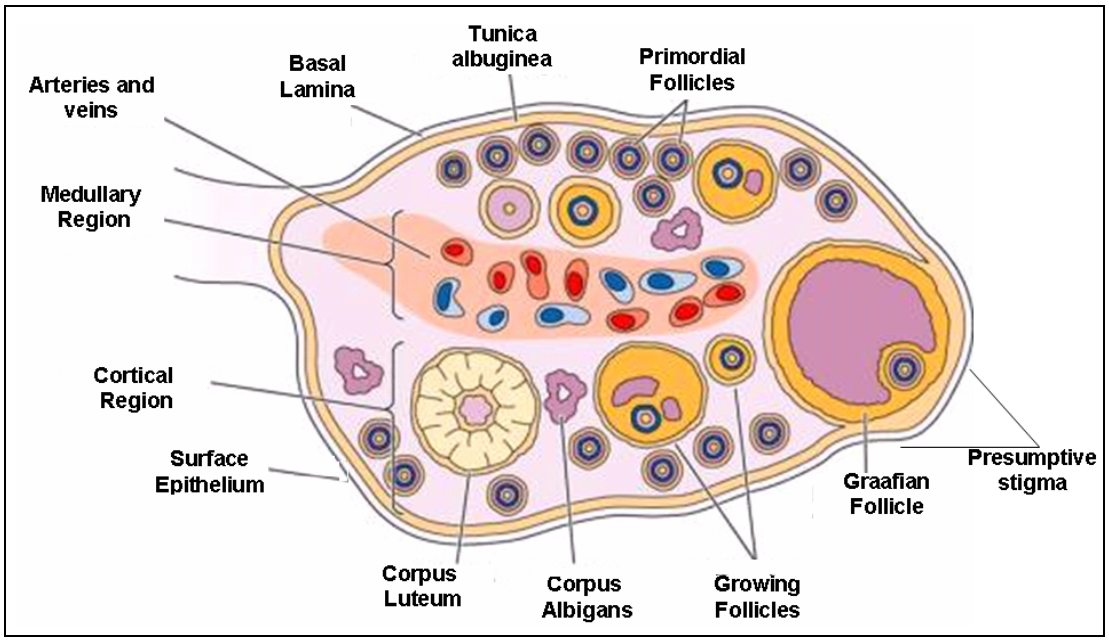

Figure 1.2: The human ovary. Follicles of different developmental stage as well as corpus luteum and corpus albigans are spread in cortical zone. Arteries and veins are located within the medulla. Tunica albuginea surrounds the ovarian stroma and is separated from the surface epithelium through a basal lamina. The Graafian follicle is protruded at the rupture point prior to ovulation and formation of the stigma. Downloaded and adapted after http://www.mc.vanderbilt.edu/histology/labmanual2002/labsection3/FemaleRepTract03_files/image002.jpg (2008).

1.2.3 Endocrinology of the human ovary-The menstrual cycle

It is well-established that the menstrual cycle consists of two phases: the follicular phase and the luteal phase. These two phases are separated by ovulation after which fertilisation can occur; however, if fertilisation fails to happen, menstruation follows the luteal phase of the menstrual cycle and this signifies the initiation of a new reproductive cycle (Fig. 1.3). The follicular phase is the stage of the menstrual cycle when final follicle and oocyte maturation occur and is oestrogen dominant, whereas during the luteal phase, the corpus luteum is formed, secreting progestogens and thereby preparing the uterus to host the blastocyst. As noted above, during follicle growth, steroidogenically quiescent primordial follicles pass through the primary, pre-antral, antral and ovulatory stages where different cell layers are recruited. Whereas early follicular development is independent of hormone secretion, transitions from the antral follicle to an ovulatory follicle and from the ovulatory follicle to the corpus luteum require a coordinated paracrine, autocrine and endocrine dialogue among ovarian, pituitary and extrapituitary factors. Importantly, different cell layers possess different steroidogenic profiles that alter at each stage and interact with each other and with the oocyte, leading to a fertilisable oocyte. Essentially, the ovarian surface also contributes to these processes, though its constitution to the steroid milieu of the ovary has not been well studied. Notably, the distinct steroidogenic status at each stage is the result of differential expression of the enzymes that are involved in the steroidogenic pathway. Simultaneously, secretion of ovarian sex steroid hormones affect uterine morphology and function that also alter between oestrogenic proliferative (follicular) and progestogenic secretory (luteal) phases of the menstrual cycle (Johnson & Everitt 2000b) (Fig. 1.3).

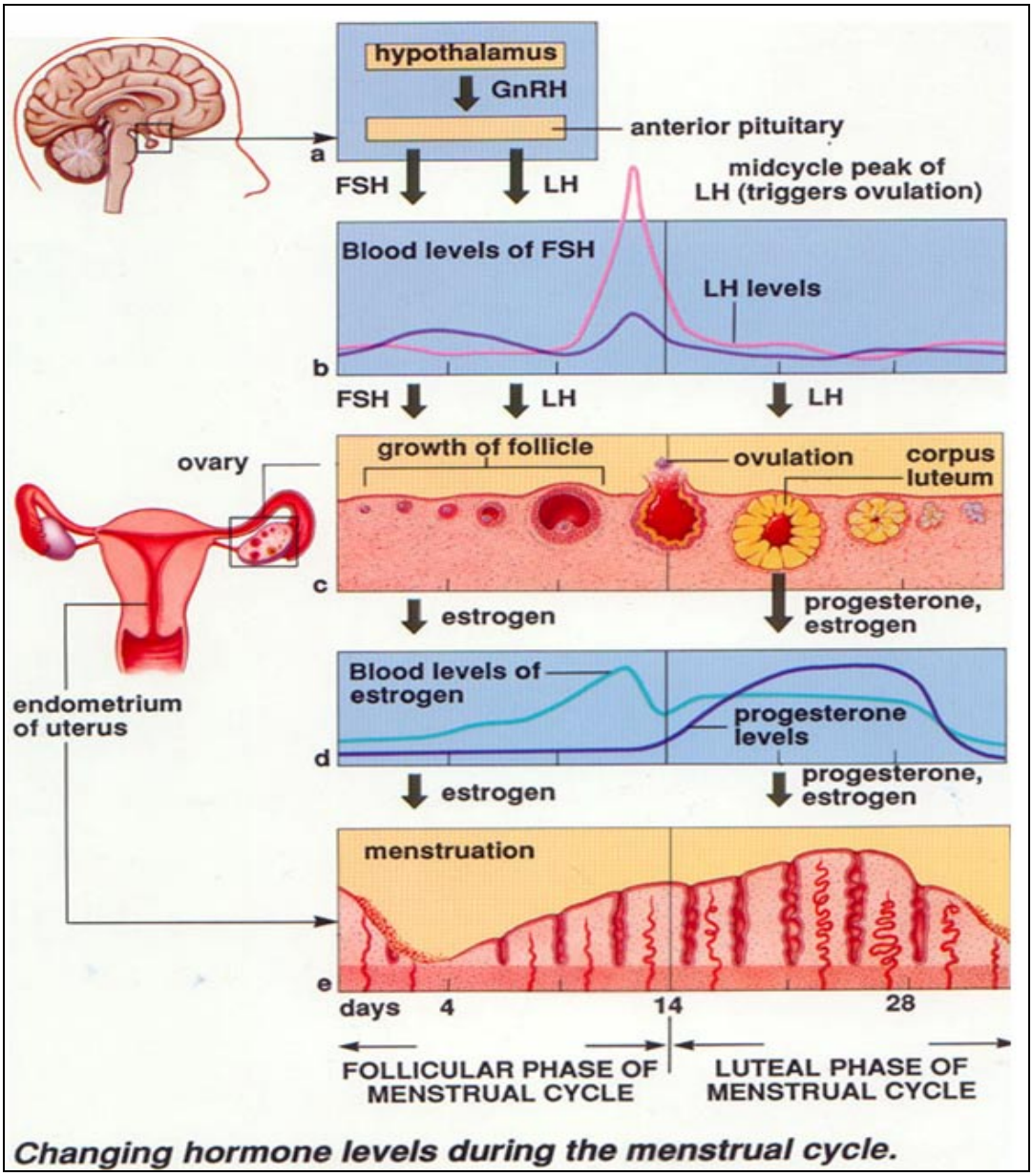

Figure 1.3: Menstrual cycle. Periodical hormonal secretion during different stages of the menstrual cycle. Feedback loop mechanisms among hypothalamus, pituitary, ovary and uterus. Follicular phase is oestrogenic-dominant under the control of pituitary FSH and LH, whilst luteal phase is progestogen-secreted following LH-induced oestrogen elevation. Downloaded from http://www.soc.ucsb.edu/sexinfo/images/05-07-Menstrual.jpg (2008).

1.2.3.1 Endocrinology of the follicle and the corpus luteum

i) The follicular phase of the menstrual cycle

Sex steroid hormone secretion in the ovary is mainly coordinated by pituitary hormones, namely follicular stimulating hormone (FSH) and luteinising hormone (LH), that determine the follicular phase of the menstrual cycle. In the antral follicle, internal thecal androgen synthesis is stimulated by the action of LH, which binds to the LH receptor (LH-R) expressed in the outer theca layer of the follicle. Furthermore, FSH binds to the FSH receptor (FSH-R) expressed in granulosa. Subsequently, gonadotrophin-receptor binding up-regulates intracellular 3'-5'-cyclic adenosine monophosphate (cAMP) levels which, in turn, serves as second messenger for the induction of the cytochrome P450 aromatase (P450arom) enzyme in the ovarian granulosa (Hillier 1994, Strauss 1999). This enzyme facilitates the conversion of androstenedione secreted in theca interna cells to oestrogens. Intriguingly, androstenedione generation in theca cells is governed by a 17β-HSD enzyme that is constitutively expessed in theca cells and is not under hormonal influence (Bogovich & Richards 1984). High FSH-driven aromatase activity within the pre-ovulatory follicle leads to the expression of LH-R in granulosa, a critical event during the selection of the follicle that will be competent for ovulation and fertilisation (pre-ovulatory follicle). Subsequently, following the LH surge, elevation of oestradiol secretion in granulosa cells ensues, triggering ovulation and final oocyte maturation. In contrast, the rest of the follicles are FSH-deficient and undergo atresia (McNatty *et al.* 1979b, 1979c). Ovulation follows between 36 and 38h after the LH surge, 24 and 36h after the oestradiol elevation and around 12h after the LH peak (Clement 1994). During ovulation, the follicle wall is ruptured and is differentiated into a highly vasculaturised endocrine gland, the corpus luteum, a procedure referred to as luteinisation. As mentioned above, the corpus luteum is characterised by its high capacity to secrete progestogens, the hormone that maintains pregnancy.

ii) The luteal phase of the menstrual cycle

Endocrine activity and survival of the corpus luteum are subject to its sensitivity to LH, an effect mediated by small luteal cells (lutein-theca), and its subsequent competence for progesterone secretion from the large luteal cells (lutein-granulosa) (Cooke 1988). Progesterone output is controlled by luteotrophic factors which differ between species. LH and chorionic gonadotrophin (CG) are the major luteotrophic factors that control human corpora lutea maintenance through binding to LH-R expressed in both types of lutein-cells (Johnson & Everitt 2000c, Rice 1966). Furthermore, FSH has been shown to promote human corpus luteum survival, by suppressing apoptotic signals (Matsubara 2000). Besides progesterone, the corpus luteum has the dynamics to synthesise oestrone, oestradiol and androstenedione (Le Maire 1968). In addition, several cytokines such as transforming growth factor-β1 (TGF-β1) and macrophage colony-stimulating factor (M-CSF) have been shown to promote luteinisation and protect corpus luteum from senescence (Matsubara 2000).

In a non-fertile cycle, the life span of the human corpus luteum is between 12 and 14 days. The corpus luteum then degenerates, enabling new follicles to grow. At the beginning of the late luteal phase, progesterone levels fall sharply due to the attenuated responsiveness of the lutein-cells to the luteotrophic signals (Bukovsky *et al.* 1995) and lutein-cells undergo atresia and ischaemia, a process known collectively as luteolysis. In ruminants and likely in primates, this event is thought to be a result of the multiple effects exerted by the prostaglandin $PGF_{2\alpha}$ which is secreted by both the uterus and luteal cells and suppresses progesterone biosynthesis (Bennegård *et al.* 1991), probably through down-regulation of the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) (Duncan *et al.* 1998), the steroidogenic enzyme responsible for the formation of active progesterone (prog/P4) from its precursor pregnenolone (Preg/P5) (Samuels *et al.* 1951). Importantly, $PGF_{2\alpha}$ mediates its effects through binding to the cognate receptor expressed in large luteal cells (Cooke 1988). Simultaneously, an increase in immune cell populations that secrete cytokines such as interferon-γ (INF-γ), tumour necrosis factor-α (TNF-α) and

the chemokines interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) occurs (Bornstein *et al.* 2004, Matsubara 2000). Subsequently, release of matrix metalloproteinases (MMPs) ensues, culminating in breakdown of the extracellular matrix (ECM) and thus destruction of the corpus luteum (Bornstein *et al.* 2004, Duncan 2000). Intriguingly, the reduced responsiveness of the corpus luteum to LH/CG is not due to LH-R attenuation in lutein-cells, because it is well-established that LH-R expression in these cells persists, albeit at lower levels, during corpus luteum degradation (Duncan *et al.* 1998). Instead, it has been proposed that endothelial cells, in the regressing human corpus luteum, sharply express LH-R thereby avoiding the transport of plasma LH/CG to the extravascular space of this tissue (Bukovsky *et al.* 1995). Consequently, steroid secretion by theca- and granulosa-lutein cells is further abrogated (Best & Hill 1995), culminating in lutein-cell senescence. As a result, the corpus luteum regresses and becomes an inactive, small, avascular gland before being absorbed into the stromal component of the ovary. On the other hand, in case of pregnancy, LH-R expression is absent in endothelial cells and therefore LH/CG-responsive lutein-cells maintain their progestogenic-producing capacity (Bukovsky *et al.* 1995), a parameter critical for corpus luteum survival and maintenance of pregnancy.

1.2.3.2 Endocrinology of the human ovarian surface epithelium

Whereas the biology and endocrinology of the ovarian follicle and the corpus luteum are well-established, little is known about the endocrinology of the human ovarian surface epithelium. As stated above, increased interest of this miniscule compartment of the ovary rose sharply in the last decade when it was realised that it was the main source of ovarian cancer (Auersperg *et al.* 2001). Experimental data have demonstrated that this epithelial cell population has all the machinery for hormonal synthesis and reception, suggesting a role of this cell surface in ovarian function. However, whether the OSE hormonal milieu undergoes periodical changes within the menstrual cycle is not known due to lack of animal models and restrictions in human tissue availability on the necessary scale for any profound study.

There is evidence that hOSE is positive for gonadotrophin-releasing hormone (GnRH) receptor (Kang *et al.* 2000), FSH-R (Choi *et al.* 2002) and LH-R (Kuroda *et al.* 2001). Although gonadotrophins have been proved mitogenic and inhibit apoptosis (Choi *et al.* 2002, Kuroda *et al.* 2001), they do not appear to be involved in the secretion of steroids from hOSE as opposed to the other ovarian compartments indicated above (Ivarsson *et al.* 2001a, Okamura *et al.* 2003). However, intracrine steroid generation has been predicted by an oligonucleotide microarray analysis showing mRNA expression for all the enzymes that are necessary for local steroid biosynthesis (Rae *et al.* 2004b). In particular, this study showed the presence of transcripts for P450 cholesterol side chain cleavage, (P450scc), steroid acute regulatory protein (StAR) and 3β-HSD, highlighting that the production of progesterone *in situ* is possible. Moreover, hOSE were positive for the mRNA of 17β-HSD enzymes that are involved in oestrogen and androgen biosynthesis as well as for steroid sulfatase (STS), the enzyme responsible for oestrone formation from circulating oestrone sulphate (Rae *et al.* 2004b). Also, transcripts for aldoketoreductases (AKR), the enzymes that inactivate steroids, were also present (Rae *et al.* 2004b). Collectively, these data indicate that the hOSE layer is a site of steroid metabolism, possessing all the machinery to control steroid bioavailability and therefore hOSE might well contribute to ovarian steroidogenesis. Therefore, it is of interest that hOSE cells can produce *de novo* steroid precursors from cholesterol along with the use of precursors which are produced by extra-ovarian or adjacent ovarian cells (Rae *et al.* 2004b, Rembiszewska & Brynczak 1985). The importance of steroid biosynthesis along with the major steroidogenic pathway is discussed in more detail in following sections of this Chapter.

A growing body of evidence has shown that hOSE is also a site of steroid reception as revealed by the presence of androgen receptor (AR), progesterone receptor (PR-A, PR-B), oestrogen receptors (ER-α, ER-β) (Edmondson *et al.* 2002, Lau *et al.* 1999, Li *et al.* 2003, Mukherjee *et al.* 2005) and glucocorticoid receptor-α (GR-α) (Rae *et al.* 2004a), all members of the nuclear receptor superfamily of ligand-dependent transcription factors. Therefore, an understanding of steroid and

gonadotrophin signalling in hOSE may prove pivotal for the functionality and regulation of this compartment. Additionally, several studies have reported that hormonal signalling is directly linked with the aetiology of ovarian cancer. This is discussed in more detail in later sections of this Chapter.

1.2.4 Ovulation

1.2.4.1 Ovulation as a process

Ovulation is defined as the process where the oocyte proficient for fertilisation is released. This process is tightly controlled and in humans occurs on a monthly basis and lasts between 36 and 40 hours. By the mid-luteal phase of the menstrual cycle very few follicles (less than four) reach a 4-5mm diameter to compete for ovulation in the next reproductive cycle. By the late follicular phase of the menstrual cycle only one will reach a 15-25mm diameter, encompassing the pre-ovulatory or Graafian follicle. The latter starts to migrate within the ovarian stroma and protrudes at the ovarian surface at the so-called follicle rupture point or stigma (Fig. 1.4). Following local proteolytic enzyme and prostaglandin secretion and action, the ovarian surface epithelium, the tunica albuginea and the theca externa at the apex of the ovulation site degenerate and the follicle wall breaks allowing oocyte release into the peritoneal cavity. The stigma now becomes ischaemic, being enclosed by follicular fluid, granulosa cells, fibrin, connective tissue cells and blood. Eventually, the wounded ovary is repaired involving intensive healing processes essential for the preparation of the ovary to host the next ovulatory cycle (Clement 1994, Okamura *et al.* 2003).

1.2.4.2 Ovulation as an acute inflammatory reaction

It has been almost 30 years since Espey first proposed that ovulation is an acute inflammatory reaction (Espey 1980). This hypothesis was generated based on similarities in physical and biochemical events occurring during ovulation with those occurring during acute inflammatory cascades in typically injured tissues.

Inflammation occurs when the homeostasis of a tissue is disrupted causing a trauma. During this process, the first line of defence system, the so-called innate immune cells such as dendritic cells, natural killer cells, macrophages, basophils, eosinophils and mast cells, are activated and induce histamine production that subsequently promotes infiltration of leukocytes into the injured tissue. The latter, in turn, trigger secretion of soluble immune mediators such as cytokines, chemokines and matrix proteases that surround the trauma. Concomitantly, this results in the activation of the adaptive immune cells such as B and T lymphocytes that counteract inflammation and initiate tissue remodelling and regeneration of the harmed tissue. Therefore, controlled cell proliferation and cell death of genetically damaged cells ensue, allowing extracellular matrix deposition and re-epithelisation and as such tissue homeostasis is restored (de Visser & Coussens 2005).

In the ovary, the LH surge prior to follicular rupture increases the vascularity and capillary permeability. As in inflamed tissues, the latter is partially mediated by histamine and prostaglandins, induction of which is achieved by the LH-induced cAMP elevation. The primary source of histamine is immune cell populations such as leukocytes and macrophages as well as proliferating thecal fibroblasts that surround the Graafian follicle. Ovarian follicular wall collapse is achieved by proteolytic enzymes such as plasminogen activator (PA) that is mainly released by white blood cells and proteases such as trypsin that are mainly secreted by fibroblasts (theca externa) (Espey 1980, 1994). Following wounding-induced inflammatory mediator secretion, cell proliferation occurs as part of the healing process (Brannstrom & Norman 1993, Buscher *et al.* 1999). Reconstruction of the wounded tissue involves regeneration of the connective tissue, components of which such as collagen and gelatine are secreted by fibroblasts.

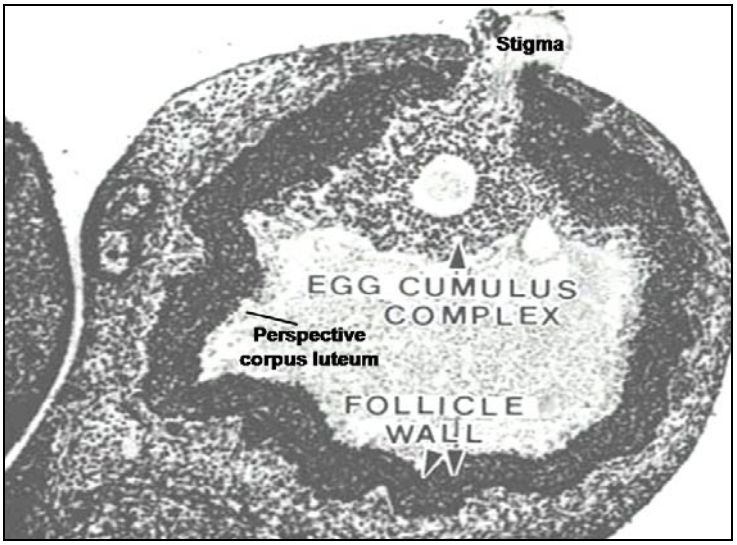

Figure 1.4: Stigma. The follicle ruptures at the ovulation point or stigma. The follicle wall breaks, the OSE sloughs and the cumulus-oophorus complex is released in the peritoneal cavity. Luteinisation ensues to form the corpus luteum of the luteal phase of the menstrual cycle. Adapted from http://www.endotext.org/female/female1/figures1/figure20.jpg (2008).

1.2.4.3 Inter-relationships between cytokine system and ovulation

Cytokines are polypeptide molecules that are naturally secreted at specific stages of the menstrual cycle such as ovulation and menstruation. As aforementioned, ovulation is an acute inflammatory event closely associated with tissue damage followed by an anti-inflammatory repair process (Brannstrom & Norman 1993, Buscher *et al.* 1999). During this process, the Graafian follicle is surrounded by cytokines secreted either by the steroidogenic follicular cells (granulosa and thecal cells) themselves or by immune populations such as leukocytes (macrophages, lymphocytes and neutrophils) that have migrated to the site of follicle rupture (Brannstrom & Norman 1993, Brannstrom *et al.* 1994, Buscher *et al.* 1999). Along with the cytokines that promote the rupture of the follicle causing the wounded tissue, cytokines that induce healing mechanisms are also present in both the Graafian follicle and the corpus luteum. For example, the IL-1 system has been demonstrated to have a major role in intra-ovarian function. IL-1α secretion in the follicular fluid can induce IL-6 secretion that in turn stimulates follicular rupture through

stimulation of a prostaglandin-associated proteolytic pathway. This deleterious pro-inflammatory effect can be counteracted by the IL-1 receptor antagonist (IL-1Ra) (Buscher *et al.* 1999). Granulocyte-macrophage-colony-stimulating factor (GM-CSF), TNF-α, IL-8 (Buscher *et al.* 1999, Zhao *et al.* 1995) and IL-4 (Hashii *et al.* 1998) are also present in the follicular fluid of the peri-ovulatory follicle.

Intriguingly, leukocyte-induced cytokines can affect steroidogenesis as well. Strikingly, TNF-α and IL-1α have been reported to abolish steroidogenesis and more precisely progesterone synthesis at the peri-ovulatory period (Terranova & Rice 1997), whereas IL-4 has been reported to induce progesterone synthesis (Emi *et al.* 1991, Hashii *et al.* 1998). Another striking example of cytokine-endocrine interactions is the stimulation of glucocorticoid synthesis through induction of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in response to IL-1α pro-inflammatory effects in the granulosa cells of the peri-ovulatory follicle (Tetsuka *et al.* 1999). Notably, it was proposed that this effect represents an efficient mechanism for the ovary to counteract inflammatory-associated damage and promote rapid healing (Hillier & Tetsuka 1998). It is therefore clear that cytokines are indispensable factors for the fulfilment of ovarian functions such as steroid formation, differentiation and proteolysis prior to and through peri- and post-ovulation.

1.2.4.4 Role of the human ovarian surface epithelium in ovulation: Injury and repair

As mentioned earlier, the human ovarian surface epithelium or hOSE is a single cell layer that lines the ovary and separates it from the other peritoneal structures. OSE facilitates the transportation of materials to and from the peritoneum (Anderson *et al.* 1976). Importantly, it is believed to be an integral part of the ovulatory process, since ovulation is abrogated after surgical removal of the ovine OSE *in vivo* (Colgin & Murdoch 1997) and frog OSE *in vitro* (Schuetz & Lessman 1982), maybe due to the fact that, besides the follicle and the surrounding leukocytes, OSE secretes components such as proteases, extracellular matrix components and cytokines that have a critical role in follicular wall breakdown.

Following follicular rupture, the OSE at ovulation sloughs (stigma) undergoing an injury and repair process that involves cell degradation typical of apoptosis and proliferation (Fig. 1.4) (Murdoch 1995, Osterholzer *et al.* 1985). Apoptosis is thought to be induced by prostaglandins that are locally secreted when the time of ovulation approaches (Ackerman & Murdoch 1993) followed by intensive cell proliferation and remodelling to heal the trauma during the luteal phase of the menstrual cycle (Murdoch 1994). The exact time and stage of the luteal phase of the menstrual cycle when cell proliferation and repair (healing) of the stigma take place is not clear and further studies in animal and human models are required to further investigate that. Also, it is yet to be established whether cell proliferation and repair happen at the same phase of the luteal phase of the menstrual cycle. However, it is understandable that post-ovulatory wound healing is essential such that the ovary can host the next ovulatory cycle (Clement 1994, Okamura *et al.* 2003).

As part of the injury, it has been shown that OSE cells produce enzymes such as cyclooxygenase type 2 (COX-2) that facilitate prostaglandin secretion which in turn trigger collagen breakdown (Rae *et al.* 2004a). Interestingly, this mechanism is probably mediated by elevated sex steroid levels that surround the pre-ovulatory follicle (Bjersing & Cajander 1975). OSE cells, at least in sheep, are also positive for urokinase plasminogen activator (uPA), expression of which is triggered by the elevated gonadotrophin levels in the pre-ovulatory follicle (Colgin & Murdoch 1997). Plasminogen activator of the OSE and pre-ovulatory follicle synergise to induce TNF-α secretion that in turn potentiates local MMP (MMP-1 and MMP-2) expression along with microvascular coagulation, both involved in acute inflammation, nuclear DNA damage and cellular necrosis (Larrick & Wright 1990), thus leading to stigma formation (Murdoch & McDonnel 2002). Importantly, not only does inhibition of TNF-α action suppress ovulation but it also impedes ECM degeneration (Johnson *et al.* 1999, Murdoch *et al.* 1997).

Besides immune cells and the follicle, OSE itself has been proved capable of secreting cytokines. Hence, it has been shown that OSE produces significant amounts

of pro-inflammatory GM-CSF, CSF, M-CSF, IL-1α, TNF-α, IL-18 and IL-6 as well as T lymphocyte-associated cytokines such as IL-10 (Burke *et al.* 1996, Lidor *et al.* 1993, Wang *et al.* 2002b, Ziltener *et al.* 1993). Furthermore, OSE is positive not only for chemokines such as MCP-1 and IL-8 but also for growth factors such as epidermal growth factor (EGF) and TGF-β1 (Burke *et al.* 1996, Ziltener *et al.* 1993). They have also the ability to respond to many of these agents. Importantly, OSE is positive for IL-4 and INF-γ receptors in the absence of *de novo* capacity to produce their ligands (Burke *et al.* 1996, Ziltener *et al.* 1993). Such findings point to paracrine, autocrine and intracrine roles of this cellular monolayer in ovulation and wound healing cycles.

Importantly, there are implications from epithelial-mesenchymal transitions as well as cell separation, migration and mitotic activity during OSE regeneration (Auersperg *et al.* 1994b, Ziltener *et al.* 1993). Although OSE has a mesothelial phenotype with mesenchymal and epithelial features during a non-ovulatory cycle, it acquires either stromal or ectopic epithelial characteristics when it is submitted to a regenerative process *i.e* after follicle rupture. Intriguingly, the latter phenotype also seems to occur in cell culture, indicating the plasticity of these cells and their responsiveness to environmental factors (Auersperg *et al.* 2001). During regenerative processes, OSE cells are frequently invaginated in the stroma, forming inclusion cysts (clefts) (Fathalla 1971, Murdoch 1994, Okamura *et al.* 2003) (Fig. 1.5). Physiologically, these entrapped cells can differentiate into fibroblast-like cells, and this could be an efficient mechanism to prevent neoplastic transformation (Auersperg *et al.* 2001). Alternatively, such inclusion cysts are believed to give rise to pathological conditions such as benign malignancies and metaplasias (Fathalla 1971). Under such circumstances, OSE loses its mesemchymal-stromal character and is committed to an epithelial phenotype by developing Müllerian duct derived epithelia behaviour. Therefore, it can be transformed to tubal, endometrium or uterine cervix epithelia that give rise to serous, endometrioid or mucinous epithelial ovarian cancers (EOC) respectively (Kaku *et al.* 2003, Mittal *et al.* 1995).

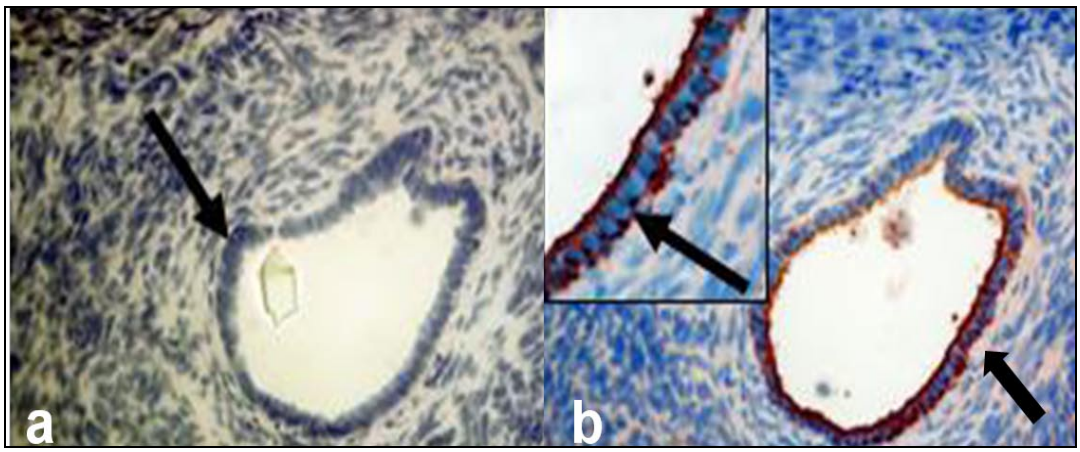

Figure 1.5: Inclusion cyst. Epithelial cells are entrapped in the ovarian stroma of a pre-menopausal ovary (a). Epithelial character of these cells was confirmed by staining with epithelial-specific cytokeratin 8 (b). Adapted after Rask *et al.* (2006).

1.3 Relevance of the Physiological Function of hOSE in the Development of Epithelial Ovarian Cancer

Development of ovarian cancer is a multifactorial disease involving a complex network of molecular and cellular pathways that all synergise to promote an oncogenic phenotype. Depending on the origin of the tumour, ovarian cancer is classified as surface epithelial-stromal tumour or epithelial ovarian cancer (EOC) when it arises from the OSE, sex cord-stromal ovarian tumour when it is of mesonephric or mesenchyme origin (granulosa, theca, stromal cells) and germ cell tumour when it derives from primordial germ cells (Fig. 1.6). Over 85% of ovarian cancers are of epithelial origin with ovarian sex cord-stromal and germ cell tumours being rarer cases (Chen *et al.* 2003). From now on, further references to ovarian cancers will relate to tumours of epithelial origin, because the other two groups of ovarian cancers, albeit equally important, are outwith the focus of this thesis.

It is widely accepted that EOC arises from the clonal expansion of a single transformed cell (Ozols *et al.* 2004). It is commonly believed that hOSE is the origin of EOC, because this tissue is very dynamic and undergoes cyclical hormone changes and continuous regeneration during reproductive life. Progression of the disease is probably the result of accumulating premenopausal genetic errors that have escaped from the surveillance of the molecular repairing mechanisms, involving cell cycle-, apoptosis- and differentiation-associated genes (Murdoch 1998, Murdoch *et al.* 2001, Ozols *et al.* 2004, Zeimet & Marth 2003). Pre-neoplastic transition probably takes place later at menopause when the hormonal environment has radically changed and basic DNA-repairing mechanisms probably alter or switch off.

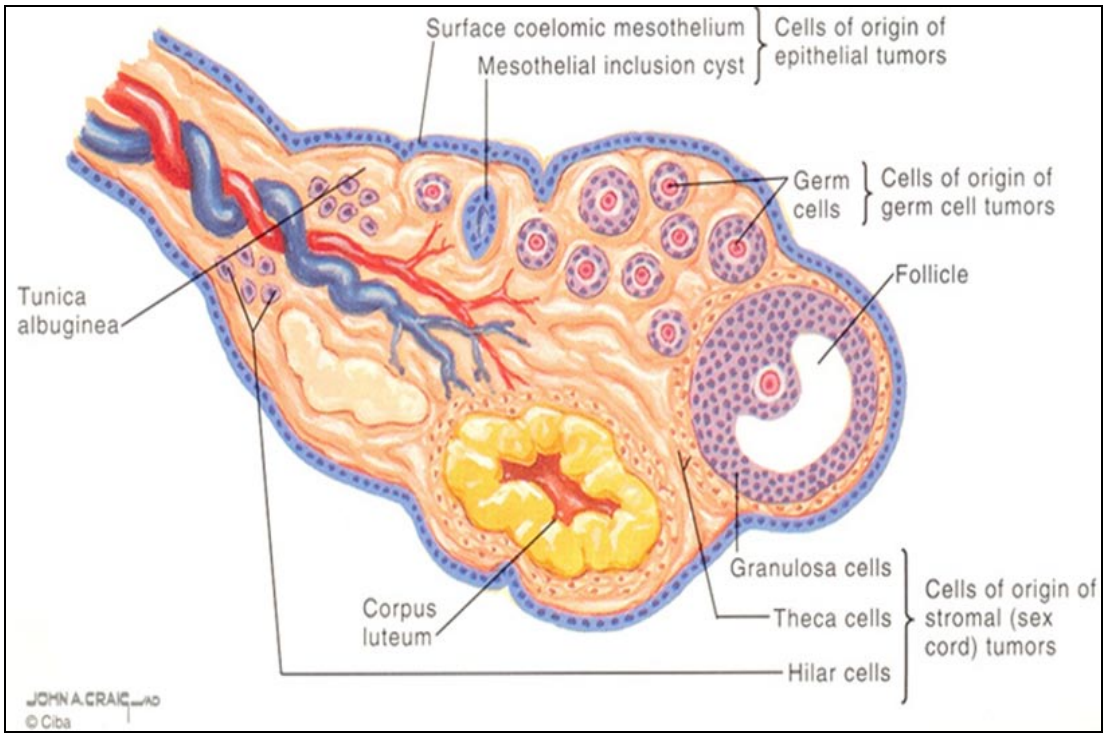

Figure 1.6: Classification of ovarian cancer depending on the origin of the tumour. Epithelial ovarian cancer arises from the OSE, germ cell tumours originate in primordial germ cells and sex cord-stromal tumours arise from ovarian stromal, granulosa and thecal cells. Designed by John A Craig. Kindly provided by Professor Charles H. Blomquist, University of Minesota, Mineapolis, USA.

1.3.1 Epidemiology of ovarian cancer- Links with natural reproductive events

Along with positive association of menopause with the development of the disease, another risk factor is the family history, albeit it accounts only for 4-5% of all cases (La Vecchia 2001). Exposure to cosmetics and chemicals such as talc and asbestos are believed to increase the risk although their role in the development of the disease is controversial (La Vecchia 2001, Langseth *et al.* 2008 , Merritt *et al.* 2008, Muscat & Huncharek 2008, Ness *et al.* 2000b, Wong *et al.* 1999). Endometriosis (Merritt *et al.* 2008, Modugno *et al.* 2004, Ness *et al.* 2000b) and pelvic inflammatory disease (PID) (Ness & Cottreau 1999, Risch & Howe 1995) are considered to be positively associated with the development of ovarian cancer as well. Oestrogen replacement therapy for longer than ten years is believed to increase the risk of ovarian cancer three-fold (Lacey *et al.* 2002); however, the Million Women Collabotrators study showed that this increased risk falls after termination of oestrogen therapy (Beral 2007). Polycystic ovarian syndrome (PCOS) and the associated risk of ovarian cancer is controversial (Balen 2001, Schildkraut *et al.* 1996). Reproductive and hormonal factors such as multiparity (Adami *et al.* 1994, Gwinn *et al.* 1990, Hankinson *et al.* 1995, Riman *et al.* 2004, Risch *et al.* 1994), lactation (Risch *et al.* 1994, Siskind *et al.* 1997), oral contraceptive use (Collaborative group on epidemiological studies of ovarian cancer 2008, Franco & Duarte-Franco 2008, Gwinn *et al.* 1990, Hankinson *et al.* 1995, Ness *et al.* 2000a), tubal ligation and hysterectomy (Green *et al.* 1997, Kreiger *et al.* 1997, Ness *et al.* 2000b) are thought to decrease the risk. A summary of the factors that are believed to play a role in the aetiology of EOC are illustrated in Table 1.1.

1.3.2 Hypotheses for tumour initiation/progression

Based on the epidemiological data referred to above, at least three hypotheses have been generated to explain the development of EOC from natural processes the hOSE undergoes during fertile life. It should be mentioned that these hypotheses are interrelated and thus not mutually excluded and all jointly are probable contributing factors in the development of the disease.

1.3.2.1 Incessant ovulation hypothesis

Fathalla first proposed that the continuous ovulations that the female experiences during reproductive age may have profound implications in the development of epithelial ovarian neoplasms. EOC can derive from OSE cells entrapped in the ovarian stroma, forming inclusion cysts as a result of the repair of the ovulatory stigma. Expansion of a genetically damaged cell can result in oncogenesis (Fathalla 1971). Indeed, post-ovulatory healing of the surface epithelium involves exposure to cytogenotoxic agents that can harm the cell integrity (Ozols *et al.* 2004). The latter has been extensively shown in several basic research studies using both human and animal models. Prolonged *in vitro* subculture of rat ovarian surface cell lines display abnormal karyotypes characterised by clonal aberrations, unbalanced rearrangements and numerical losses as a result of malignant transformation (Godwin *et al.* 1992, Testa *et al.* 1994). *In vitro* prolonged culture of mouse OSE followed by intraperitoneal injection of late passage cells resemble Stage III and IV ovarian cancers and yield an aneuploid karyotype (Roby *et al.* 2000). Human OSE cells in *in vitro* culture display epithelio-mesenchymal transitions through subculturing, mimicking *in vivo* ovulation-associated regeneration. However, hOSE cell cultures from patients with family history (*i.e.* with BRCA-1/BRCA-2 mutations) appear more committed to an epithelial phenotype after long-term propagation, showing a susceptibility of those cells to a pre-neoplastic phenotype probably developed after continuous ovulations (Dyck *et al.* 1996). Moreover, *in vivo* injection of the mutagen 7,12-dimethylbenz(a)anthracene to induce carcinogenesis combined with gonadotrophins to mimic ovulation-affected OSE, results in epithelial lesions in rat ovaries. Intriguingly, the stage of the ovarian neoplasia is more severe with increasing doses of the mutagen (Stewart *et al.* 2004). Also, the animals generate point mutations in *p53* and *ki-Ras* genes (Stewart *et al.* 2004), approaching what has been observed in late stage human ovarian malignancies (Aunoble *et al.* 2000, Berchuck *et al.* 1994, Mayr *et al.* 2006, Wang *et al.* 2004). Nonetheless, it is worth noting that mutant p53 overexpression in ovarian cancer is likely more a cause of general genetic instability due to accumulated

genetically harmed cells rather than a direct effect of ovulation, suggesting a role in the progression rather than the initiation of the disease (Schildkraut *et al.* 1997). It has also been reported that 8-oxoguanine adducts were more frequent in post-ovulatory relative to inactive OSE in ewes, ovine, humans and hens (Murdoch & Martinchick 2004, Murdoch *et al.* 2001, Murdoch *et al.* 2005). Addition of indomethacin to suppress ovulation reduced 8-oxoguanines in the OSE cells of ovulation (Murdoch *et al.* 2001). Collectively, these and other studies further support the concept that random expansion of irreparable non-committed to death cells arising from post-ovulatory repair can lead to an oncogenic phenotype (Fathalla 1971, Murdoch 2005).

The incessant ovulation hypothesis is supported by epidemiological studies suggesting that ovulatory-inhibitory events such as pregnancy, multiparity, breast-feeding and oral contraception reduce the risk of EOC (see 1.3.1 for references). Moreover, it is of interest that ovarian neoplasms from puberty onwards, when the female has experienced repetitious ovulatory cycles, are of epithelial origin as opposed to rare germ-cell and stromal-sex cord ovarian tumours that constantly appear before the menharge age (Fathalla 1971). Additionally, EOC rates increased during the 20th century where it has been estimated that women release about 400 oocytes in their lifetime as opposed to the previous century when women had more children and thus wasteful ovulations were less abundant (Banks *et al.* 1993). On the other hand, anovulatory situations such as PCOS and infertility are thought to promote rather than reduce EOC risk, although this has not been confirmed in every study (see 1.3.1 for references). Also, another caveat of this hypothesis is that the progestin-only pill that does not impede ovulation has been reported more protective than the combined (oestrogens with progestogens) ovulation-suppressive pill (Risch 1998, Rodriguez *et al.* 1998).

1.3.2.2 Gonadotrophin and sex steroid hormone hypothesis

As referred to above, ovulation is a controlled reaction tightly driven by pituitary gonadotrophins as well as ovarian steroids, androgens and oestrogens, that in turn result in progesterone secretion peri- and post-ovulatory. Therefore, ovulation could indirectly lead to the development of EOC through the secretion of reproductive hormones prior to and after ovulation. As noted, the ovarian cell surface at the site of rupture as well as the cells entrapped in the stroma (inclusion cysts) are exposed to high levels of gonadotrophins that derive from the peri-ovulatory follicle. Subsequently, gonadotrophins trigger oestrogen secretion that in turn signifies ovulation. Thus, gonadotrophins and oestrogens equally could give rise to neoplastic transformation of OSE (Cramer & Welch 1983) (Fig. 1.7).

Elevated gonadotrophin levels in follicle-depleted post-menopausal ovaries, when the majority of ovarian cancer cases occur suggest that they impact upon OSE (Cramer & Welch 1983). Several studies have shown that FSH and LH/hCG signal via their cognate receptors in the ovarian cell surface, ovarian pre-neoplastic and cancer cell lines (Choi *et al.* 2002, Kuroda *et al.* 2001, Parrott *et al.* 2001, Syed *et al.* 2001, Zheng 1996). All of these studies have shown cytoproliferative effects of gonadotrophins in the ovarian cell surface, attesting to a direct link with the development of ovarian cancer. Numerous mechanisms have been described to explain gonadotrophin cell growth stimulatory effects. It has been shown that FSH exerts its effects through rapid activation of the mitogen-activated protein kinase (MAPK)-responsive Elk-1 transcription factor (Choi *et al.* 2002), leading to activation of oncogenic pathways (Choi *et al.* 2004) in ovarian pre-neoplastic and neoplastic cells. In particular, overexpression of FSH-R in OSE cell lines resulted in extracellular signal-regulated kinase (ERK)1/2 activation and accompanied with increased expression of growth factor receptors and oncogenes such as EGF-R, Her2/neu receptor, c-myk (Choi *et al.* 2004), catenin-β1 and cyclin G2 (Ji *et al.* 2004). Interestingly, FSH has been established to impact upon EGF-R activity, whilst LH impacts upon EGF-R mRNA stability. Moreover, differential FSH- and LH-

induced EGF-R cytoproliferative effects have been shown to be mediated by ERK1/2 and phosphatidyl-inositol-3-kinase (PI-3K) pathways (Choi *et al.* 2005, Gubbay *et al.* 2004). Furthermore, recent data are demonstrative that these cytoproliferative effects can be successfully blocked by supplementation of ovarian cancer cells with EGF-R inhibitors and EGF, thereby sensitising the cells to taxol-associated cell death pathways (Cao *et al.* 2008). The IL-6/signal transducers and activators of the transcription protein **(**STAT)-3 signalling pathway has been also reported to drive FSH and LH mitogenic activity in ovarian epithelial immortalised and ovarian cancer cell lines (Syed *et al.* 2002). Another mechanism for gonadotrophin action in ovarian cancer development is through suppression of the anti-proliferative GnRH and its cognate receptor (Choi *et al.* 2006, Choi *et al.* 2001a). A further study showed that mitogenic effects of LH on ovarian cancer cell lines are exerted through abrogation of the Fas-induced cell death pathway (Slot *et al.* 2006b). The Fas pathway has been shown to be active in the human ovarian surface epithelium as well as in ovarian cancer and it is one of the major mechanisms of the ovary to induce cell apoptosis (Ghahremani *et al.* 1998, van Haaften-Day *et al.* 2003). Consistently in the rat ovary, pro-apoptotic associated Fas ligand and bax molecules have been mainly detected in OSE at the apex of ovulatory follicles. Active caspase-3 has been also observed in post-ovulatory OSE cells (Slot *et al.* 2006a)**.** Along with cytoproliferative effects, it has been suggested that gonadotrophins may promote cell invasion and adhesion through stimulation of proteolytic enzymes such as MMP-2 and MMP-9 and inhibition of tissue inhibitors of MMPs (TIMPs), TIMP-1 and TIMP-2 (Choi *et al.* 2006). An angiogenic effect of FSH on OSE has been also shown (Wang *et al.* 2002a).

Positive links of increased LH and FSH levels agree with the protective role that breast-feeding has against EOC (See 1.3.1 for references). Previous reports have suggested that anovulation-related breast-feeding is a result of decreased LH and FSH (Chubak *et al.*, 2004; McNeilly 2001). Moreover, ovarian cancer cysts contain more abundant FSH and LH levels relative to benign tumours, suggesting a positive link of gonadotrophins with EOC that should not be underestimated (Krämer *et al.*

1998). Nonetheless, whereas the vast majority of studies support that FSH and LH mediate cell proliferation and survival, there are conflicting reports. In primary hOSE cell cultures from pre-menopausal and post-menopausal women, FSH suppressed cell proliferation, especially in the case of confluent cell cultures from post-menopausal women (Ivarsson *et al.* 2001b). The reason for this discrepancy is not known, though different cell culture systems and treatment concentrations may be responsible for these inconsistencies (Choi *et al.* 2007). Alternatively, this effect could also mimic *in vivo* natural OSE cell death (stigma) occurring as a part of oocyte release when gonadotrophin levels are elevated (Choi *et al.* 2007, Murdoch 1995).

Another mechanism through which gonadotrophins can contribute to the development of EOC could be indirectly through exposure of the ovulatory OSE to gonadotrophin-induced oestrogens. Oestrogenic 17β-HSD5, the enzyme for the intracrine formation of oestrogens, as well as ERs (ER-α and ER-β) are expressed in both OSE and neoplastic ovarian cells (Blomquist *et al.* 2002, Cardillo *et al.* 1998, Hillier *et al.* 1998, Lau *et al.* 1999, Li *et al.* 2003, Nagayoshi *et al.* 2005, Rae *et al.* 2004b). Oestrogen levels are 10,000 times higher in the peri-ovulatory OSE than their circulating levels (Risch 1998). Moreover, another oestrogen peak occurs at the luteal phase of the menstrual cycle when post-ovulatory OSE healing is thought to take place (Clement 1987, 1994, Murdoch & Van Kirk 2002). It appears, therefore, that ovarian cytoproliferation is associated with a high oestrogenic environment. Intriguingly, ER-α appears to be the functional receptor with little responsiveness of the ER-β. For example, in primary cancers and cancer cell lines, the ER-α/ER-β ratio appeared higher than in normal OSE cells, revealing a positive link of ER-α and EOC (Li *et al.* 2003). In support of this, ER-α but not ER-β has been shown to be positively up-regulated by the thyroid hormone, triiodothyronine (T_3), an inflammatory gene-associated surrogate (Rae *et al.* 2007). Moreover, a cDNA microarray study showed twenty-eight genes to be affected by oestradiol treatment in PEO1 ER-positive ovarian cancer cells and these effects were mediated by ER-α and not the ER-β isoform and were blocked when the anti-oestrogen tamoxifen was

applied (O'Donnell *et al.* 2005). Oestradiol has been also shown to induce the IL-6/STAT3 mitogenic signalling pathway in human immortalised and ovarian cancer cell lines (Syed *et al.* 2002) as well as the c-myc oncogene in NIH:OVCAR-3 cancer cell line (Chien *et al.* 1994). Inhibition of apoptosis in response to oestradiol has been also demonstated in sheep OSE and notably this effect appeared to be non-genomic since it was not blocked by tamoxifen (Murdoch & Van Kirk 2002b). Up-regulation of the extracellular matrix component fibulin-1 has been also shown to be triggered by oestradiol in EOC cell lines, attesting to a possible metastatic and invasive role of this steroid hormone (Clinton *et al.* 1996). Establishment of ovarian neoplasms after exposure of guinea pigs to diethylstilbestrol and oestradiol has been also demonstrated (Silva *et al.* 1998). Overall, it appears that a rich oestrogen environment either in hOSE itself or in the surrounding ovarian components exerts a cell growth effect and this is possibly a contributing factor in EOC development (Fig.1.7). This agrees with the epidemiology that claims a positive link of oestrogen replacement therapy and the development of the disease that can be reversed when oestrogen supplementation stops (see 1.3.1 Section for references).

Along with oestrogens, a series of studies have demonstrated cytoproliferative effects of androgens in normal OSE and EOC through their binding to the cognate androgen receptor (AR) (Edmondson *et al.* 2002, Lau *et al.* 1999, Syed *et al.* 2001). It has been established that androgens may impact upon OSE through intracrine and/or paracrine mechanisms (Risch 1998). Androgenic 17β-HSD has been demonstrated in the ovarian surface epithelium, suggesting a capacity of this monolayer to form androgens *in situ*. Moreover, androgens are secreted daily by growing follicles suggesting a possible paracrine interaction thereof with the OSE of inclusion cysts or of the ovarian surface (McNatty *et al.* 1979a, Risch 1998) (Fig. 1.7). It is also widely established that the post-menopausal ovary, that is at greater risk for the development of EOC, retains the steroidogenic capacity to produce androgens (Havelock *et al.* 2006, Laughlin *et al.* 2000). Previous studies have consistently shown increased AR protein (Cardillo *et al.* 1998, Kühnel *et al.* 1988) and co-activators (Shaw *et al.* 2001) in ovarian cancer. Another study revealed that

5α-dihydrotestosterone (5α-DHT) can reverse the TGF-β1-induced inhibition of cell growth (Berchuck *et al.* 1992, Choi *et al.* 2001c) not only in primary normal hOSE cells but also in several primary cancer cells. Importantly, the same study showed that 5α-DHT positively mediated AR co-activators such as androgen receptor-associated protein 70 (ARA-70) and steroid receptor co-activator-1 (SRC-1) (Evangelou *et al.* 2000). Furthermore, guinea pigs displayed benign epithelial cysts, small papillary neoplasms and glands in the stroma in response to testosterone treatment. Notably, testosterone serum levels were approximately 500 times higher than in control animals (Silva *et al.* 1997).

It is obvious therefore that androgens may play an indispensable role in the aetiology of ovarian cancer and this agrees with epidemiological studies that associate androgen-related events such as PCOS, hirsutism and acne with EOC (Schildkraut *et al.* 1996). Moreover, it has been demonstrated that the contraceptive pill that protects against development of the disease attenuates testosterone and dehydroepiandrosterone sulphate (DHEA-S) formation (Murphy *et al.* 1990, van der Vange *et al.* 1990).

As discussed in Section 1.3.1, pregnancy, multiparity and oral contraception are associated with a reduced risk of development of ovarian cancer. All these anovulatory events are associated with high levels of progestogens. Notably, it has been proved that the progestin-only contraceptive pill that does not impede ovulation is more protective than the combined pill (Risch 1998, Rodriguez *et al.* 1998). Moreover menopausally, progesterone secretion appears attenuated (Havelock *et al.* 2006). Collectively, progestogens appear to protect from the emergence of the disease.

A sequence of basic and epidemiological studies has been conducted to investigate further this hypothesis and identify the mechanisms through which progesterone acts upon the surveillance of the ovary and more precisely of OSE. Progesterone mainly signals through binding to the nuclear PR, both isoforms of which (PR-A, PR-B) have been shown to be expressed in the ovarian cell surface and

ovarian cancer cells (Lau *et al.* 1999, Li *et al.* 2003, Mau *et al.* 1999, Mukherjee *et al.* 2005). Epidemiological studies have recognised the deletion of PR in a high percentage of ovarian cancer specimens and loss of PR is associated with a poor prognosis for the disease (Abd-Elaziz *et al.* 2005, Cardillo *et al.* 1998, Langdon *et al.* 1998, Lau *et al.* 1999, Li *et al.* 2003). Also, ovarian cancer development is more common in women with progesterone deficiencies and progesterone receptors are absent or genetically altered in EOC (Ho *et al.* 2003). Moreover, in primary hOSE and ovarian cancer cell lines, it has been demonstrated that both oestrone and oestradiol negatively regulate PR and this effect can be blocked by the ER antagonist ICI 182780. Additionally, high doses of progesterone increased caspase-3 activity and inhibited cell mitogenesis. This effect was further enhanced in co-treatment with ICI 182780 and was abolished in co-treatment with the PR antagonist RU486 (Mukherjee *et al.* 2005). In accordance, another study showed an enhancement of OSE cell proliferation after addition of another PR antagonist, Org 31710 (Ivarsson *et al.* 2001a). Additionally, oestrogen-induced ovulation in the mouse resulted in a stratified appearance of the ovarian epithelium as opposed to a normal appearance of the OSE after co-administration with progesterone (Gotfredson & Murdoch 2007). Likewise, the apoptotic rates of macaque OSE appeared three-fold higher in progestin-induced animals than in those administered with both oestrogens and progestogens (Rodriguez *et al.* 1998). Consistently, oestradiol cytoproliferative effects were counteracted by progesterone administration in sheep (Murdoch & Van Kirk 2002b). Commonly, progesterone appears to inhibit cell growth and trigger apoptosis in the OSE and this might be an efficient mechanism to nullify the clonal expansion of genetically spoilt cells.

The mechanisms through which pro-apoptotic effects of progesterone are exerted have been investigated. As mentioned above, the ovary is subject to wound healing and repair cycles of the OSE in response to ovulation and this can lead to genetic instability of the ovarian cell surface. It has been shown that progesterone triggers apoptosis to attenuate potential genetic damage of the post-ovulatory ovarian surface. In ewes and sheep, progesterone treatment resulted in a decline of 8-

oxoguanine adducts through induction of polymerase-β and poly(ADP-ribose) polymerase (PARP) increase, both involved in DNA repair. These effects appeared to be progesterone-specific and exerted at the transcriptional level as it was revealed by supplementation of RU486 and actinomycin D respectively (Murdoch 1998, Murdoch *et al.* 2001). Moreover, reports suggest that progesterone exerts its apoptotic and anti-proliferative effects through up-regulation of the tumour suppressor p53 protein (Murdoch & Van Kirk 2002b). The latter has been also seen in human ovarian cancer cell lines, where the progesterone exerted anti-proliferative and apoptotic effects through arrest of cells at the G1 phase of the cell cycle and concomitant stimulation of p53 mRNA (Bu *et al.* 1997). This possibly facilitates the DNA repair machinery to restore or remove any genetically instable cells and protect against clonal expansion of any transformed cell (Murdoch & Van Kirk 2002). Also, there is evidence that progesterone promotes cell apoptosis through activation of caspase-8 and caspase-3 pathways that in turn stimulate the Fas/Fas-ligand apoptotic pathway (Syed & Ho 2003).

In summary, it is obvious that reproductive hormones can be beneficial or destructive for the integrity of the ovarian cell surface. Therefore, tightly controlled cytoproliferation, cell survival and cell apoptosis are subject to a balanced dialogue among reproductive hormones to minimise the chance of any fatal scar occuring on the ovarian cell surface.

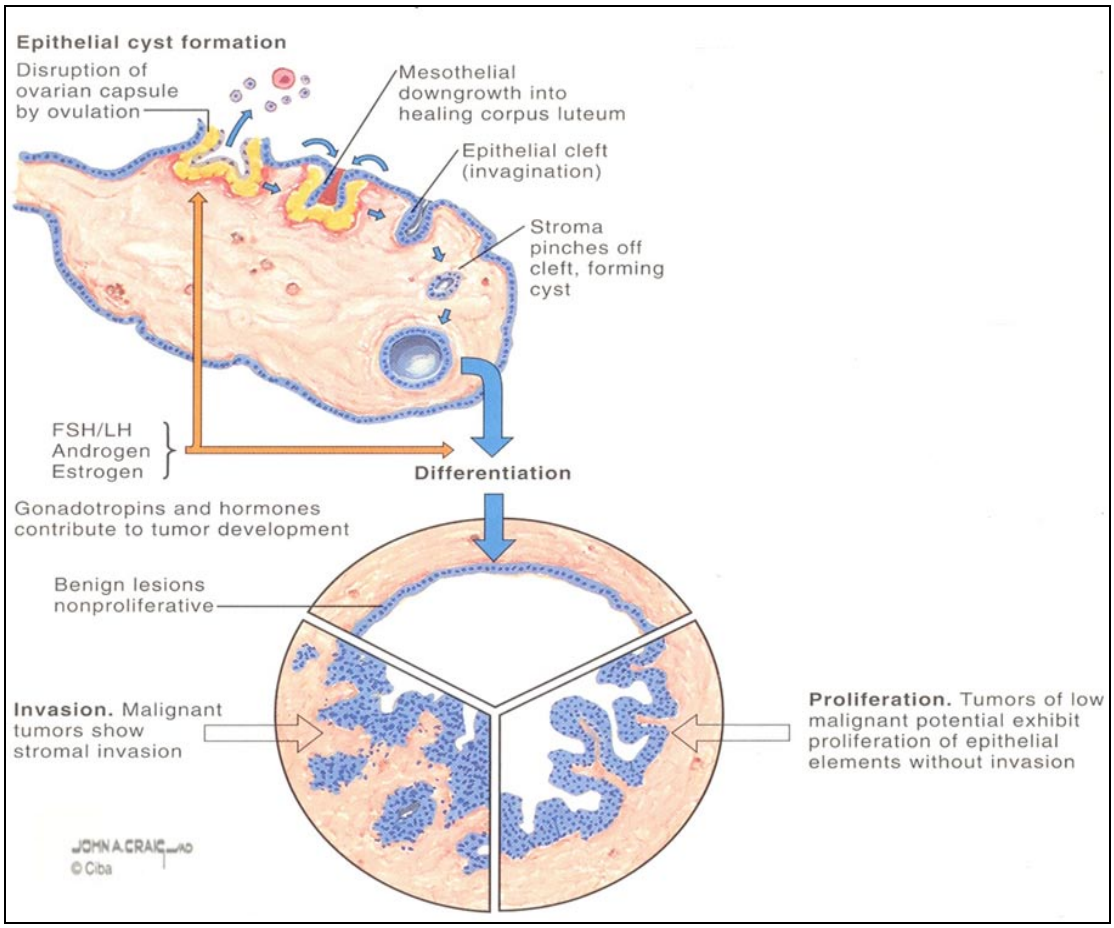

Figure 1.7: Impact of reproductive hormones on inclusion cysts and/or hOSE. During ovarian post-ovulatory repair of hOSE, sloughed OSE cells are invaginated in the stroma forming inclusion cysts. Both cysts and hOSE are exposed to gonadotrophins and steroid hormones during the menstrual cycle. As such, cells differentiate and this may lead to neoplastic transformation. Designed by John A Craig. Kindly provided by Professor Charles H. Blomquist, University of Minesota, Mineapolis, USA.

1.3.2.3 Inflammation hypothesis

As recalled throughout this chapter, ovulation along with ovulation-associated hormones impact upon the ovarian cell surface and these are considered risk factors for the development of EOC. On the other hand, anovulatory events as well as suspension or stimulation of individual reproductive hormone secretion appears essential for prevention of EOC. It was aforementioned that ovulation has a local inflammatory basis, involving secretion of immune mediators that positively or negatively affect local hormonal milieu and vice versa (Espey 1980, 1994, Terranova & Rice 1997). Moreover, factors that are associated with development of the disease such as asbestos, talc and endometriosis may act in the OSE through induction of inflammatory responses. Conversely, a protective role of hysterectomy and tubal ligation could be subject to the blockage of inflammatory effects of these factors. Jointly, all these led to the idea that inflammatory events triggered either naturally by ovulation or environmentally by extraovarian factors may impact upon OSE integrity, thereby initiating EOC (Ness *et al.* 2000b).

i) Inflammation and cancer

Links between inflammation and cancer are not a novel concept. The role of inflammation in the development of cancer was first hypothesised in 1863 by Rudolf Virchow who recognised the infiltration of leukocytes into tumour tissues, suggesting initiation of tumour progression at sites of chronic inflammation (Balkwill & Mantovani 2001). As a general concept, the development of cancer is the result of perturbations of the innate and adaptive immune balance, thus resulting in excessive tissue remodelling that in turn places the tissue in a continuous oxidative stress, increasing therefore the susceptibility of cells to DNA and protein alterations (de Visser & Coussens 2005). For example, breast carcinoma is characterised by excessive infiltration of macrophages that lead to increased vasculature and angiogenesis of the tumour (Leek *et al.* 1996, 1999). Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are part of the tumour milieu and contribute to the growth and spread of the tumour (Balkwill & Mantovani 2001, Balkwill *et al.* 2005).

Notably, TNF-α secretion by epithelial or stromal cells can directly and indirectly promote carcinogenesis of hepatocytes and skin through induction of the nuclear factor kappa B (NF-κB) inflammatory pathway. This effect was not apparent in the TNF-α knockout mouse. Besides, tumour progression was attenuated with anti-TNF-α therapy as well as induction of inhibitor kappa B (IκB) - an intermediate NF-κB suppressor - at later stages of hepatitis tumour development (Arnott *et al.* 2002, Pikarsky *et al.* 2004).

Another contributing factor to cancer progression is T lymphocyte-associated cell dysfunction and this appears to be the case in invasive renal (Das *et al.* 2008) and squamous head and neck cancers (Gastman *et al.* 2000). Intriguingly, FasL-induced T-cell apoptosis as well as inhibition of T-lymphocyte anti-inflammatory effects by T regulatory (Treg) cells have been proposed as candidate mechanisms for the tumour to escape T cell surveillance (Das *et al.* 2008, Gastman *et al.* 2000, Hori *et al.* 2003, Woo *et al.* 2002).

Furthermore, a classic pathway to spoil immune balance is through mutations and polymorphisms of genes that control immune regulation and responses such as cell survival, tissue remodelling and angiogenesis, all factors that contribute to the development of cancer (Mantovani 2005). For example, gastric cancer is related to IL-1 polymorphisms (El-Omar *et al.* 2000). Strikingly, attenuated skin neoplastic development was observed in mast cell-deficient mice, largely due to down-regulation of MMPs and thus restriction of angiogenic and cytoproliferative effects, suggesting that tumour progression may be harnessed by altering harmful innate immune cell action (Coussens *et al.* 1999). Moreover, B and T lymphocyte deficiency in skin carcinogenic mouse model (HPV16 mouse) resulted in attenuation of blood vascularity, angiogenic vascular endothelial growth factor (VEGF)-A and MMP-9 proteins as well as epithelial cytoproliferative responses. However, injection of B-lymphocytes in HPV16 mice brought back chronic inflammation-induced carcinogenic potential, thereby attesting a dual role of adaptive immunity in cancer

progression - T cells eliminating cell invasion, whereas B cells appearing perpetrators of cancer promotion (de Visser *et al.* 2005).

Another molecule that appears to be up-regulated in neoplasias and mediated by pro-inflammatory cytokines is COX-2, a major regulator of cell survival, angiogenesis and immune control. Intriguingly, COX-2 inhibitors are considered key therapeutic targets in inflammation-related tumours such as mammary cancer (Basu *et al.* 2004, Chang *et al.* 2005).

Synoptically, it is obvious that inflammation is a critical factor of initiation, progression and invasion of tumours.

ii) Inflammation and development of epithelial ovarian cancer

Consistent with the inflammation hypothesis for the development of EOC (Ness & Cottreau 1999) along with solid evidence for a role of inflammation in many types of cancer described above, a series of studies have been performed to investigate the mechanisms through which inflammation can promote the development of EOC. Regarding this, two approaches have been used: a) elucidation of inflammation-associated events in both malignant and normal ovary and b) identification of anti-inflammatory mechanisms that could protect OSE from neoplastic transformation.

As discussed in section 1.2.4, the immune system has a key role in ovarian functions such as ovulation and steroid formation and action. It was also mentioned that hOSE is responsive to immune mediators such as cytokines which impact upon it in an autocrine and paracrine manner. Fundamentally, cytokine secretion and responsiveness to cytokines such as IL-6, IL-1, TNF-α, GM-CSF and IL-8 is retained in the ovarian tumour microenvironment, suggestive that the development of EOC is a consequence of altered local immune balance (Burke *et al.* 1996, Merogi *et al.* 1997, Nash *et al.* 1998, Obata *et al.* 1997, Szlosarek *et al.* 2006, Ziltener *et al.* 1993). Notably, it has been shown that both normal and malignant ovaries lack ‘anti-

tumour' T lymphocyte-associated cytokines such as IL-4 and IL-10, albeit that receptors for these cytokines are present (Burke *et al.* 1996), thereby implicating that secretion from adjacent cells is essential for immune homeostasis. In support of this, T lymphocyte dysfunction has been shown in ovarian carcinomas. Remarkably, it has been reported that T lymphocyte apoptosis is induced by the Fas ligand that is secreted by tumour cells. Intriguingly, this effect was reversed when caspase inhibitors were present (Rabinowich *et al.* 1998). Another candidate mechanism to eliminate T lymphocyte 'anti-inflammatory' action is by T regulatory cells that infiltrate the tumour milieu. Importantly, a positive correlation between T regulatory cells, spread of the ovarian tumour in the peritoneal cavity and poor prognosis of the disease has been demonstrated (Curiel *et al.* 2004).

An additional striking paradigm of immune imbalance in ovarian cancer is the loss of biologically active pro-inflammatory IL-18. Precisely, it has been shown that mature IL-18 which normal OSE expresses is lost in ovarian cancer (Wang *et al.* 2002b). Also, heterozygous IL-18 GC genotype was proposed as a marker in ovarian cancer invasion and metastasis (Bushley *et al.* 2004).

Another pro-inflammatory cytokine that has an indispensable role in natural ovarian processes and is a candidate for the development and progression of EOC is TNF-α (Murdoch *et al.* 1997, Murdoch 1999). TNF-α has been shown to have a role in extracellular matrix degradation during ovarian rupture, triggering therefore a series of inflammatory processes. Its capacity to impact upon OSE by mediating genes involved in membrane degradation such as uPA and MMP-9 prior to and post-ovulation reveals its possible part in OSE neoplastic transformation (Murdoch & Lund 1999, Yang *et al.* 2004). Expression of TNF-α in the apex of epithelial tumour areas and ascites along with its cytoproliferative effects have been also reported (Marth *et al.* 1996, Naylor *et al.* 1993). A proposed mechanism for TNF-α action in tumour progression and invasion is through activation of mitogenic and inflammatory signalling pathways such as Jun N-terminal kinase (JNK) and NF-κB respectively (Hagemann *et al.* 2005, Kulbe *et al.* 2005). Supplementation with

factors, such as infliximab, that suppress tumour-associated TNF-α secretion has been proposed as an immunotherapeutic modality in ovarian cancer treatment (Szlosarek *et al.* 2006). Moreover, indomethacin, a non-steroid anti-inflammatory agent that suppresses ovulation, abrogated stigma formation in ewes and reduced the cytotoxic effect of TNF-α on OSE *in vitro* (Murdoch & Lund 1999).

Further support for the positive association of inflammation with EOC also comes from studies with the IL-1α gene family, namely IL-1α, IL-1R and IL-1Ra. IL-1α has been shown to be up-regulated in the serum of patients with EOC (Zeisler *et al.* 1998). Also, an autocrine cell growth effect of IL-1α on ovarian cancer cells has been reported (Marth *et al.* 1996). In the normal ovary, IL-1α is secreted prior to and during ovulation and signals in hOSE in a paracrine and autocrine manner. A sequence of studies has proved that this proxy alters the local inflammatory environment of hOSE. IL-1α attenuated GnRH receptor mRNA expression (Rae *et al.* 2004b), through which GnRH mediates its anti-proliferative effects (Kim *et al.* 2005). Also, this surrogate reduced 3β-HSD1 mRNA that could in turn decrease local progesterone formation that has been shown to exert anti-inflammatory and apoptotic effects on OSE (Bu *et al.* 1997, Murdoch & Van Kirk 2002, Rae *et al.* 2004a, Rae *et al.* 2004b). On the other hand, IL-1α up-regulates COX-2 mRNA expression in hOSE, showing that it potentially promotes prostaglandin secretion that in turn leads to follicular wall break and rupture, events that are associated with cell damage and genotoxicity (Rae *et al.* 2004a). It also up-regulated IL-6 and IL-8 mRNAs, both secreted as part of the ovulation-associated inflammation, and also lysyl oxidase (LOX) mRNA that is involved in basement membrane deposition and this could be relevant to tissue post-ovulatory repair as well as stimulating downstream NF-κB inflammatory pathway molecules (Rae *et al.* 2004b). At the same time, IL-1α induced 11β-HSD1 mRNA and activity, the enzyme that is responsible for intracrine regeneration of anti-inflammatory cortisol from cortisone, attesting to an efficient role of the hOSE to locally alleviate tissue from cell damage and promote post-ovulatory wound healing (Rae *et al.* 2004a, Yong *et al.* 2002). Remarkably, addition of cortisol to IL-1α-treated hOSE cells *in vitro* further

stimulated 11β-HSD1 mRNA levels, revealing a positive feedback loop mechanism for cortisol regeneration (Rae *et al.* 2004a). Concomitantly, cortisol suppressed IL-1α-induced COX-2 mRNA, highlighting once more its local anti-inflammatory action in hOSE (Rae *et al.* 2004a). On the other hand, 11β-HSD2 that reduces cortisol levels by catalysing its conversion to inactive cortisone was undetectable in primary OSE cells (Gubbay *et al.* 2004, Rae *et al.* 2004a). However, 11β-HSD2 mRNA levels are significantly higher in ovarian cancer cell lines. Moreover, cancer cell lines are not responsive to IL-1α despite measurable expression of IL-1R mRNA. These data imply that 11β-HSD2 expression and lack of 11β-HSD1 induction by IL-1α stimulus might be defining features of ovarian neoplastic transformation (Gubbay *et al.* 2005).

A separate stimulus that has been recently shown to mediate inflammatory-associated genes in hOSE is the thyroid hormone, T3. In essence, T3 directly impacts upon OSE by stimulating expression of COX-2, MMP-9 and 11β-HSD1 all involved in promoting or counteracting inflammatory cascades during ovulation (Rae *et al.* 2007). These findings have direct clinical relevance, since a positive association between hyperthyroidism and EOC has been described (Ness & Cottreau 1999).

In conclusion, it is widely accepted that inflammation-associated events affect the functionality of the ovarian cell surface (*i.e* steroid production, angiogenesis and tissue remodelling) prior to and after ovulation. Immune imbalance has been also demonstrated in ovarian carcinomas. Collectively, all these imply an early need for the identification of physiological inflammatory and anti-inflammatory mechanisms in the ovarian cell surface as well as in ovarian cancer in order to develop novel molecular markers for treating or diagnosing ovarian cancer (Rae *et al.* 2004b, Rae & Hillier 2005).

In summary, it is clear that there are obvious inter-relationships among the different hypotheses (ovulation-associated inflammation, hormone-mediated inflammation and inflammation-mediated hormone signalling), and as such EOC

should be considered and investigated as a multifactorial and complex disease. A summary of all the contributing factors of the disease is illustrated in Table 1.1.

Table 1.1 Summary of factors that are involved in the aetiology of EOC

Risk factors	**Role in hOSE/EOC**
Ovulation	tissue damage/cytotoxicity/inflammation
Inflammation	wounding/tissue remodelling
Family history	epigenetics/mutations
PCOS	elevated androgens/cytoproliferation
Endometriosis	inflammation-associated
PID	inflammation-associated
Oestrogen replacement therapy	elevated oestrogens/cytoproliferation
Hyperthyroidism	chronic inflammation
Cosmetics (talc, asbestos)	chronic inflammation
FSH	cytoproliferative/invasive/adhesive
LH	cytoproliferative
T3	inflammation-associated
oestradiol	cytoproliferative
androgens	cytoproliferative
Pro-inflammatory cytokines	inflammation-associated/tissue remodelling
T regulatory-associated cytokines	T lymphocyte apoptosis
EGF	cytoproliferative
Protective factors	
Pregnancy	progesterone-associated apoptosis/anovulation
Lactation	anovulation
Oral Contraception	anovulation
Hysterectomy/Tubal ligation	blockage of inflammation-associated factors
GnRH	anti-proliferative
progesterone	apoptotic/anti-inflammatory/anti-proliferative
T lymphocyte-associated cytokines	anti-inflammatory/anti-tumourigenic

1.4 Steroid Metabolism

As detailed in the previous sections of this chapter, steroid hormones including androgens, oestrogens, progestogens and glucocorticoids have an indispensable role in the biology of the human ovarian surface epithelium. They may be beneficial or destructive for the homeostasis of the ovarian cell surface, they are involved in cell apoptosis, cytoproliferation, inflammation and tissue remodelling and they appear to be leading candidates for the development of novel strategies to diagnose and treat EOC. Given their essential role in ovarian cell surface integrity, it is timely to investigate the local mechanisms through which steroid pre-receptor formation is regulated in hOSE. It is therefore relevant to take an in-depth look and review the major steroid formation mechanisms that occur not only in the human ovary but in many if not all tissues of the body and this is the scope of this section. In particular, a focus is given to the molecular biology and intracrinology of the 3β-HSD steroidogenic enzyme, as the regulation of this enzyme in hOSE and EOC will be the major focus of the following Chapters.

1.4.1 Intracrine generation of steroid hormones

Steroid hormones are small lipid-soluble molecules that derive from cholesterol through sequential modifications that are achieved by a panel of steroidogenic enzymes (Fig. 1.9). These enzymes locally control the biosynthesis as well as inactivation of steroid hormones namely progestogens, synthesised principally in the corpus luteum and placenta, oestrogens, secreted in the granulosa cell layers of the follicle, androgens that are mainly secreted in the testicular Leydig cells but also in theca cells of the developing follicle and glucocorticoids and mineralocorticoids that are both produced in the adrenal cortex. The synthesis of steroid hormones is subject to the bioavailability of cholesterol in the circulation or in the target cell and the induction of the production of StAR protein, the enzyme that is responsible to transport free cholesterol from the cytoplasm to the inner mitochondrial membrane of the target cell, where the first enzyme, cytochrome

P450scc of the steroidogenic pathway is located (Fig.1.9). Typically, as illustrated in Fig. 1.8, steroid enzymes principally facilitate the bioavailability of active steroidal hormones that can signal in target cells through binding to cognate nuclear steroid receptors. This, in turn, leads to the dissociation of the receptor from the chaperone complex and its homo- and/or hetero-dimerisation in the nucleus. The ligand-receptor complex, then, transactivates the transcription of target genes through binding to hormone response elements (HRE) located in their promoters (5'-flanking sequence). An alternative pathway of transcription of the target genes is the reaction of the active ligands with their cognate membrane steroid receptors (whose structure resemble G-protein couple receptors or GPCRs), leading to rapid transmission of signals through signalling transduction pathways (non-genomic actions). Function and controversies about non-genomic steroid effects have been reviewed elsewhere (Hammes & Levin 2007, Losel & Wehling 2003) and are beyond the scope of this thesis.

There are two main classes of enzymes that are involved in the gonadal and adrenal steroid production: i) the cytochrome P450 haem-containing enzymes and ii) the hydroxysteroid dehydrogenases (HSDs). A major difference between the two classes is that each P450 enzyme is derived from a single gene, whilst each HSD activity exists in multiple forms, each form a product of a distinct gene, making the investigation and understanding of their regulation more complicated.

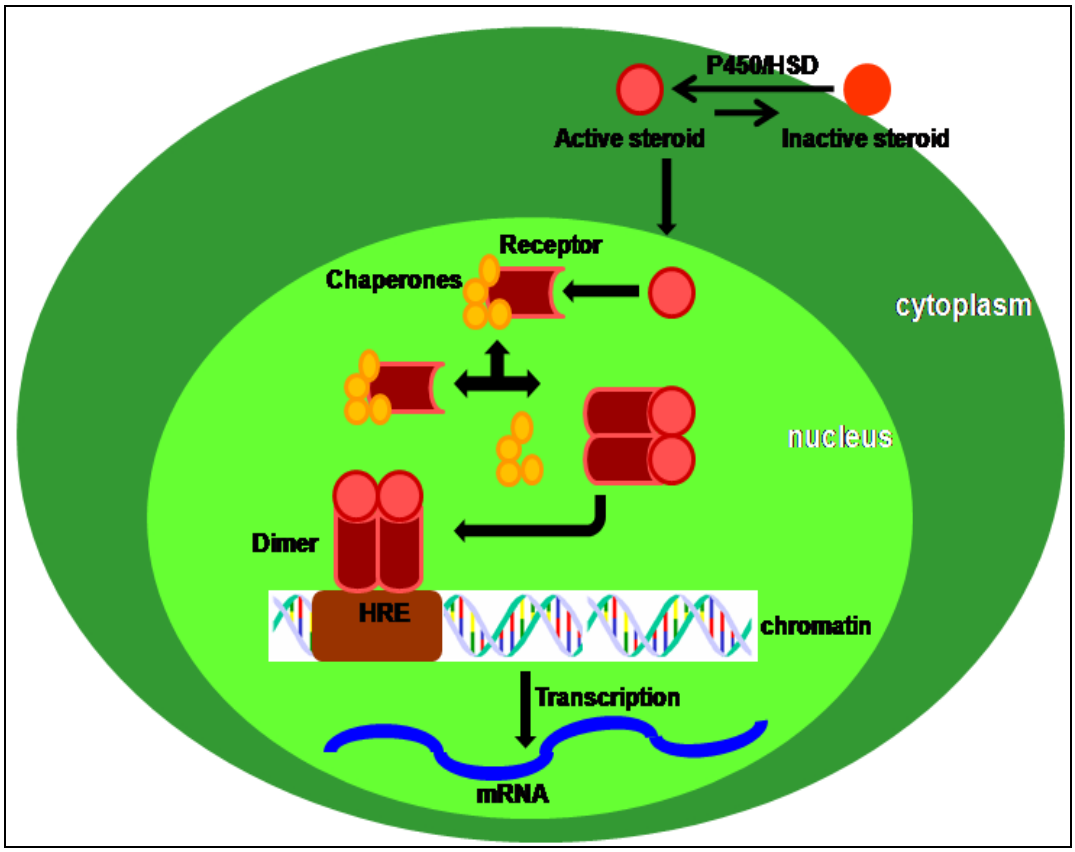

Figure 1.8: Steroid hormone action. Transactivation of nuclear steroid receptors is subject to the local availability of active steroid hormones that is determined by the catalytic efficiency of the steroidogenic enzymes (HSDs/P450s).

1.4.1.1 The cytochrome P450 haem-containing enzymes

The cytochrome P450 haem-containing enzymes are membrane-bound enzymes located either in endoplasmic reticulum (microsomes) or mitochondria. These enzymes are responsible for the hydroxylation and cleavage of steroid substrates and utilise reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-factor. Electron transfer to steroid substrate is achieved either by a mitochondrial microsomal electron transfer system. This family includes P450scc that converts cholesterol to pregnenolone and delineates the first reaction of the steroidogenic pathway, P450c17 that gives rise to DHEA or androstenedione using pregnenolone or 17α-progesterone as substrates; P450arom converts C19 androgens to C18 oestrogens, whilst P450c11 yields cortisol. Regulation of expression of P450scc and P450c11 requires the steroidogenic nuclear factor-1 (SF-1) (Lala *et al.* 1992, Morohashi *et al.* 1992). The reactions catalysed by these enzymes are illustrated in Fig. 1.9.

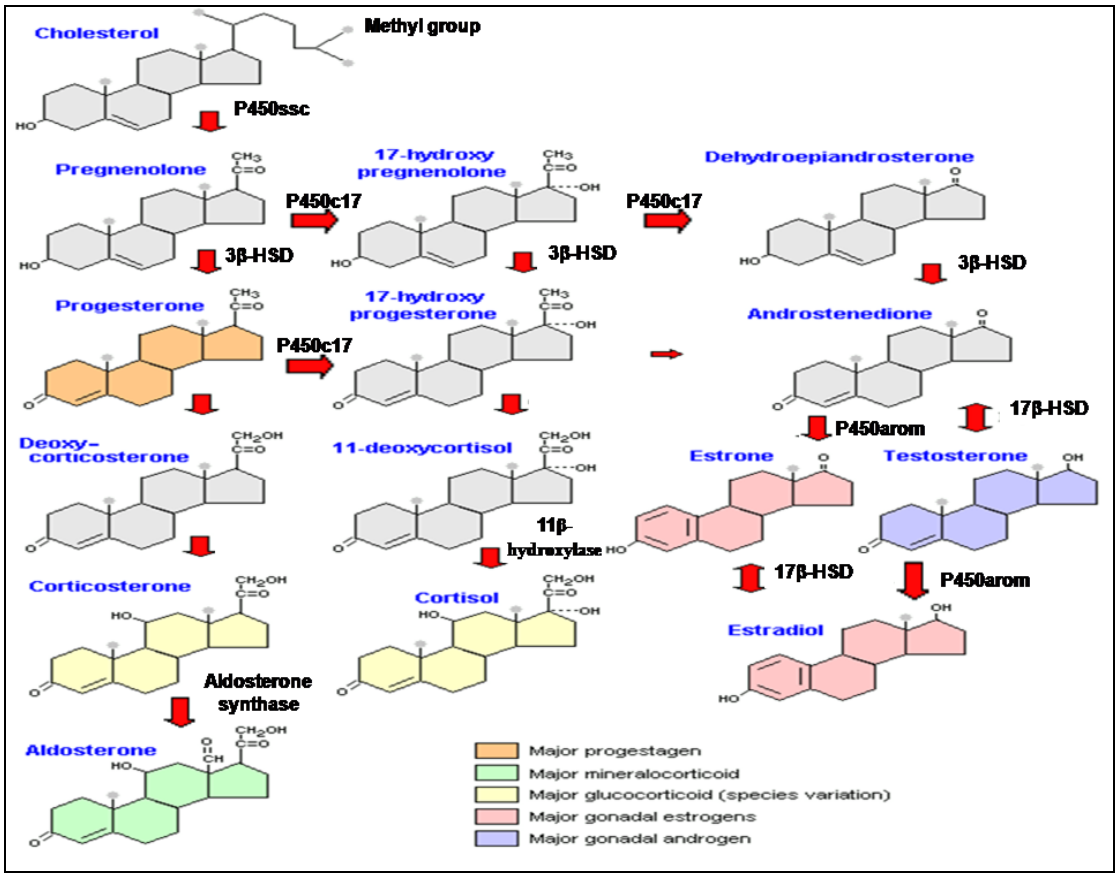

Figure 1.9: Major pathways of steroid biosynthesis. Most important steroidogenic enzymes of both P450 haem-containing and HSD enzymes. Grey coloured structures reflect main adrenal substrates that are subsequently converted to active steroid products. Modified from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/basics/steroidogenesis.gif (2008).

1.4.1.2 Hydroxysteroid dehydrogenases (HSDs)

The complexity of HSDs relates to their characteristics of not only catalysing bi-directional enzymatic reactions (dehydrogenase and/or reductase) but also to their existence as multiple isoforms with specific expression patterns and enzymatic activities in tissues and species (Penning 2003).

Milestones in the isolation of cDNA have revealed at least two distinct protein phylogenies; i) the short-chain alcohol dehydrogenase/reductase (SDR) family and ii) the aldo-keto reductase (AKR) family. The former encompasses 57 members, including the 3β-hydroxysteroid dehydrogenase/ketosteroid isomerase (3β-HSD/KSI), oestrogenic and androgenic 17β-HSDs and 11β-HSD enzymes (Krozowski 1994), whilst the latter family includes isoforms that exhibit 3α-HSD and 20α-HSD activities (including 17β-HSD5) (Deyashiki *et al.* 1995, Lacy & Dunbar 1993).

Classification of the enzymes that belong to each distinct protein family relates to similarities in protein folding patterns. The SDR family contains membrane-bound members that have 25-30% sequence homology and are multimers that consist of 25kDa monomers. Despite their low homology, they all share an α-helix/β-strand arrangement with a common motif (Gly)-xxx-Gly-x-Gly) in the N-terminus, constituting a so-called Rossman Fold $(\beta\text{-}\alpha\text{-}\beta\text{-}\alpha\text{-}\beta)_2$ (Fig. 1.10A) that is the NAD(P)(H) cofactor binding domain. Moreover, they all share a common motif (Tyr-x-x-Ser-Lys) that seems responsible for the dehydrogenase activity of the enzyme (Chen *et al.* 1993, Ensor & Tai 1991, Obeid & White 1992). Finally, the C-teminal domain varies among the members of the SDR family and it seems to relate to the binding of the different substrates (Kallberg *et al.* 2002).

The AKR superfamily includes more than 40 soluble proteins, which are monomers of approximately 34kDa. They utilise NAD(P)(H) as cofactor, although they do not have a Rossman-fold arrangement; instead a salt-linked safety-belt TIM-barrel structure $(\alpha/\beta)_8$ in the N-terminal domain allows the protein-cofactor and

protein-steroid specific interactions (Fig. 1.10B) (Penning 1997). The Tyr 55/Lys 84 pair that belongs to the Asn 50, Tyr 55, Lys 84 and His 117 tetrad appears to lead the catalytic activity of the enzyme (Schlegel *et al.* 1998). Notably, despite their differences in three-dimensional structures, site-directed mutation studies have revealed that catalytic activity of both families involves the use of Tyr/Lys pair, implicating evolution of a similar catalytic mechanism (Bennett *et al.* 1996). Distinct features of each protein family along with basic members are summarised in Fig.1.10A, B.

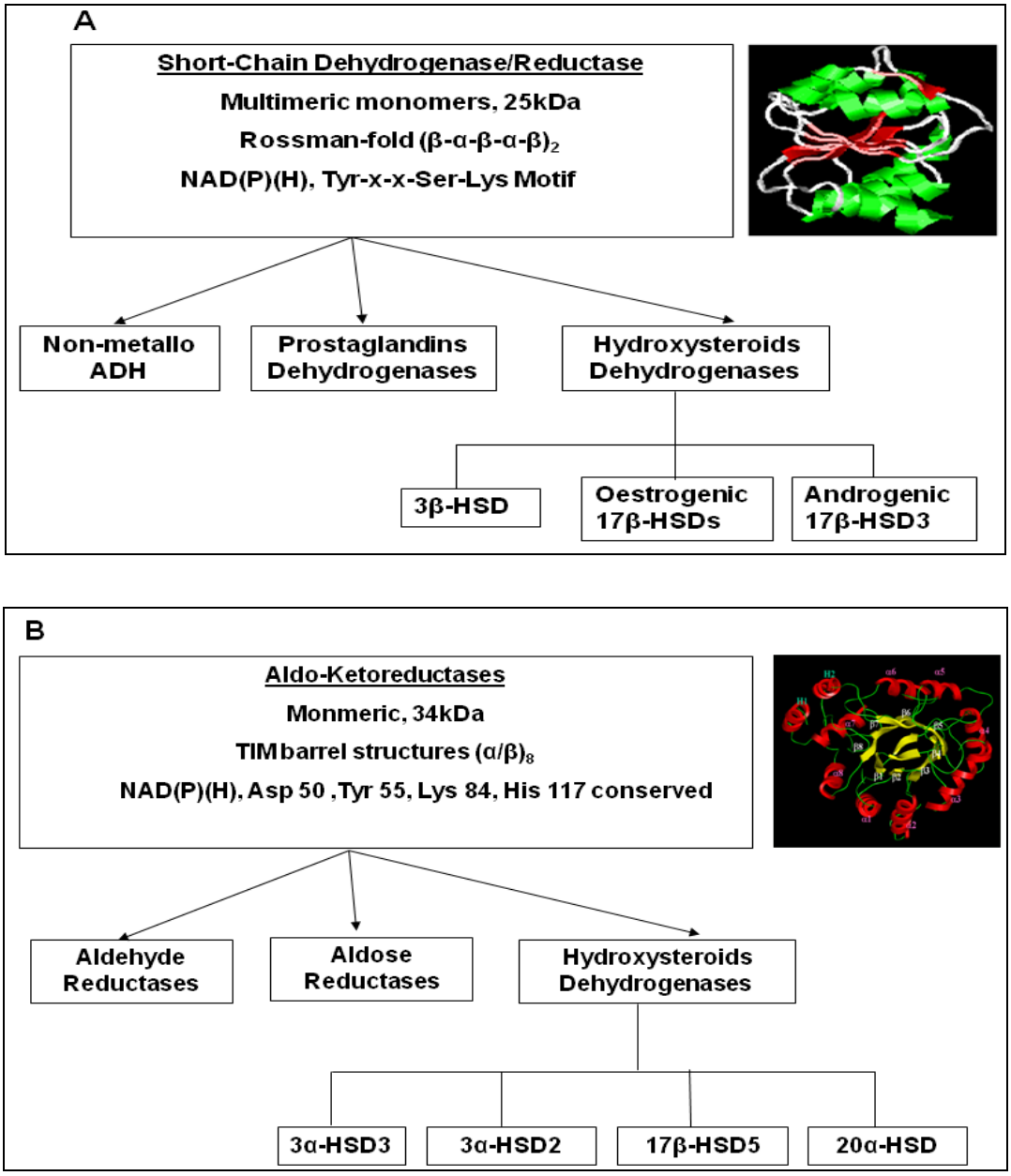

Figure 1.10: Schematic categorisation of the two major hydroxysteroid dehydrogenase families. A) Short-chain dehydrogenase/reductase members along with their basic features including Rossman-fold motif, B) Features and members of aldoketoreductase family. TIM-barrel motif is conserved among members of this HSD family. Adapted after http://www.biochem.arizona.edu/classes/bioc462/462a/NOTES/Protein_Structure/alpha_beta_domain_txt.htm (2006), http://www.med.upenn.edu/akr/akr_graphic.html (2006), Penning (2003).

1.4.2 Molecular biology and biochemistry of 3β-hydroxysteroid dehydrogenases

1.4.2.1 Biochemistry and gene structure of 3β-hydroxysteroid dehydrogenase

The 3β-HSD/KSI enzyme was first desribed in 1951 (Samuels *et al.* 1951) and catalyses the conversion of the Δ^5-3β-hydroxysteroids to the active Δ^4-3-ketosteroids. Notably, 3β-HSD/KSI is the enzyme that catalyses the first step of the conversion of steroid precursors to active steroids from cholesterol (Fig. 1.11) and therefore it controls the bioavailability of all the classes of steroid hormones, namely androgens, progestogens, oestrogens, mineralocorticoids and glucocorticoids not only in typical steroidogenic tissues but also in peripheral ones. It metabolises 3β-hydroxy-5-ene-steroids (DHEA and PREG) to 3-oxo-4-ene-steroids (androstenedione and PROG respectively) (Thomas *et al.* 1988) (Fig. 1.11).

The reaction involves two steps; following dehydrogenation of 3β-equatorial hydroxysteroids, Δ^5-3-ketosteroids products are isomerised to the *α, β*-unsaturated ketones; the first reaction is triggered by a conformational shift around a single steroid-binding site of the protein and requires reduction of NAD+ to NADH that in turn stimulates the isomerase reaction. Although 3β-HSD can convert steroid substrates to active steroids with different catalytic efficiency, enzyme activity levels are not substrate dependent (Thomas *et al.* 1988, Thomas *et al.* 1989, Thomas *et al.* 1992) (Fig. 1.11).

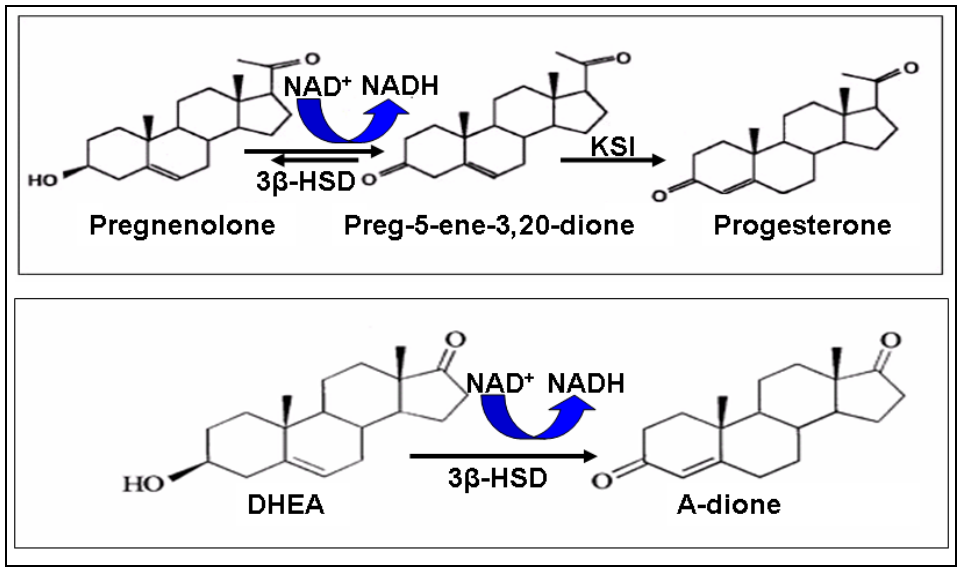

Figure 1.11: Reactions catalysed by the 3β-HSD/KSI enzyme. The conversion of pregnenolone to progesterone and DHEA to androstenedione involves reduction of binding co-factor NAD+ to NADH. Adapted after Penning (1997).

There are different isoforms of 3β-HSD/KSI with species- and tissue-specific expression patterns. The first to be isolated was from the microsomes and mitochondria of human placenta and is now called 3β-HSD type 1 (3β-HSD1) (Thomas *et al.* 1989, Thomas *et al.* 1988). The mRNA of this enzyme encodes a protein of 372 amino acids (aa) and has a molecular weight of 42kDa (Lorence *et al.* 1990). Besides progesterone and androstenedione, it is also involved in the formation and/or degradation of 5α-androstanes such as 5α-DHT. The same isoform was also identified in peripheral tissues such as skin and mammary gland. Notably, site-directed mutagenesis of 3β-HSD1 revealed that His 261 is the key residue for 3β-HSD activity, whereas Tyr 253 or Tyr 254 are responsible for the isomerase activity (Thomas *et al.* 1998). Two years later, isolation of a cDNA from human adrenal that

encodes a 371aa protein followed (Rheaume *et al.* 1991). This isoform, 3β-HSD type 2 (3β-HSD2), was also highly expressed in gonads. The *HSD3B1* and *HSD3B2* genes are located in chromosome 1p13.1, are 7.8kb and 7.7kb in length respectively, consist of 4 exons that give a 1.7kb transcript, and three introns (Berube *et al.* 1989) and they share 93.5% similarity (Rheaume *et al.* 1991). The start site of translation of both isoforms is located in exon 2 (Clarke *et al.* 1996). Gene, transcript and proteins for 3β-HSD1 and 3β-HSD2 are illustrated in Fig. 1.12.

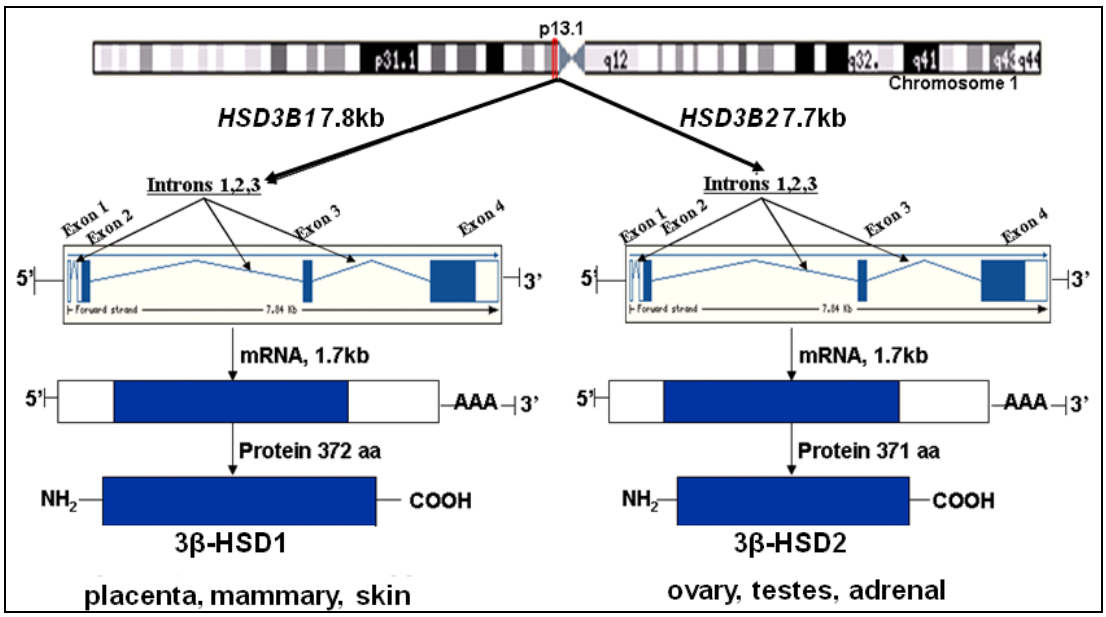

Figure 1.12: Summary of structures of *HSD3B1* and *HSD3B2* genes. *HSD3B1* and *HSD3B2* genes encoding for 3β-HSD1 and 3β-HSD2 respectively share at least 93% similarity. Adapted after ensemble databases (http://www.ensembl.org/ 2006).

A unique feature of 3β-HSDs that is absent in the other HSDs is that they have two catalytic motifs rather than one (Tyr 154-x-x-x-Lys 158) in their primary structures. Importantly, 3β-HSD1 has Tyr, whilst 3β-HSD2 has His at position 156 of this domain (Thomas *et al.* 2002a). Subcellular localisation studies revealed that 3β-HSD is a membrane-associated enzyme and is located in smooth endoplasmic reticulum and mitochondria (Thomas *et al.* 1989). Submitochondrial fractionation of

bovine adrenal showed that functional 3β-HSD protein and activity are present in the inner mitochondrial membrane and within a particulate fraction between the inner and outer membranes. 3β-HSD activity was much higher in this intermediate fraction than in the inner mitochondrial membrane and it was suggested that this might well serve the transportation of cholesterol to the inner membrane where P450scc is found and facilitating conversion of pregnenolone to progesterone (Cherradi *et al.* 1993). Kinetic studies showed that the human type 1 enzyme has 5.9-, 4.5- and 2.8-fold higher relative catalytic efficiency (maximal velocity (V_{max})/Michaelis constant (K_m)) for PREG, DHEA and 5α-DHT, respectively relative to 3β-HSD2 (Rheaume *et al.* 1991). The higher Km of 3β-HSD2 seems to be consistent with the principal expression of this isoform in steroidogenic tissues and/or cell types that *de novo* produce high levels of endogenous substrates (Labrie *et al.* 1994).

Intriguingly, five and six 3β-HSD isoforms have been identified in rat and mouse respectively (Abbaszade *et al.* 1995, Delaunoit *et al.* 1992, Lachance *et al.* 1991, Lorence *et al.* 1990, Zhao *et al.* 1991). Also, three 3β-HSD isoforms have been identified in the hamster genome (Rogerson *et al.* 1995). Single 3β-HSD genes in the bovine and canine genome only become apparent with the availability of the complete genomes of bovine and canine species (Zhao *et al.* 1989, Mendoza-Hernández *et al.* 1990). The different isoforms are numbered according to the time when they were identified and therefore the same numeral in different species does not reflect the same functional isoform. For example, mouse 3β-HSD1 is the orthologue of human type 2, whilst 3β-HSD6 is the homologue of human type 1. Notably, whereas mouse 3β-HSD types 1, 3 and 4 are dehydrogenases/isomerases, the types 4 and 5 function as 3-ketosteroid reductases and thereby they deactivate steroid biosynthesis (Abbaszade *et al.* 1995, Clarke *et al.* 1993). Rat 3β-HSD3 belongs to the latter functional group as well (Delaunoit *et al.* 1992).

1.4.2.2 Regulation of expression of 3β-HSDs

Despite their high homology, the separation of the two human 3β-HSD genes by two pseudogenes does not allow their regulation by the same promoter (McBride *et al.* 1999). Therefore, transcription of each isoform is probably regulated by different transcription factors. Hence, the most established factor that controls 3β-HSD2 expression is SF-1 with 3 consensus binding sites in the proximal promoter. Importantly, SF-1 elements are associated with cAMP and phorbol ester-induced 3β-HSD2 expression as revealed by promoter studies in human adrenocortical cells (H295R) (Leers Sucheta *et al.* 1997, Rainey *et al.* 1994). Another candidate molecule that might be involved in 3β-HSD2 transcriptional regulation is the STAT-5 transcriptional factor (Feltus *et al.* 1999, 2003).

The regulation of the mouse 3β-HSD6 (the orthologue of human 3β-HSD1) is not subject to SF-1control but is controlled by the activator protein-2 (AP-2) and the distal-less 3 (Dlx 3) that were identified in both placenta and adrenal (Peng & Payne 2002). Surprisingly, although the human 3β-HSD1 promoter has binding sites for AP-2 and Dlx3, it does not seem to be regulated by these two factors, but by the transcription enhancer factor-5 (TEF-5) and a GATA-like protein (Peng *et al.* 2004). Interestingly, 3β-HSD1 transcriptional activity is increased by cAMP, at least in choriocarcinoma JEG-3 cells (deMoura *et al.* 1997). Another candidate factor that controls human 3β-HSD1 regulation is the STAT-6 transcription factor and two STAT-6 elements have been identified in two distal sites of the gene promoter (Gingras *et al.* 1999).

1.4.2.3 Tissue-specific regulation of 3β-HSDs in female reproductive organs

As noted above, 3β-HSD1 and 3β-HSD2 arise from two distinct genes of high homology that are differentially regulated. Moreover, their catalytic efficiency differs and this may be associated with their tissue/cell-specific expression pattern. In this section a summary of the regulation of 3β-HSDs expression in distinct tissues is presented. The focus will be on the regulation in human with reference to other species when considered necessary.

i) Adrenal

The principal isoform expressed in the human adrenal is 3β-HSD2. Notably, the expression pattern of the enzyme differs between the different zones of the adult adrenal gland, highlighting distinct steroidogenic capacities of each zonal compartment (Dickerman *et al.* 1984). Thus, in the zona fasciculata (ZF) and zona reticularis (ZR) adrenocorticotropic hormone (ACTH) principally induces 3β-HSD expression, whereas 3β-HSD in the zona glomerulosa (ZG) is mainly dependent on angiotensin-II (A-II) stimulation. Intriguingly, 3β-HSD expression is suppressed in fetal human adrenal (Byrne *et al.* 1985); although *in vitro* it can be induced in response to ACTH treatment (Doody *et al.* 1990). Studies with H295R human adrenocortical cells has shown that 3β-HSD stimulation is a result of a cAMP increase triggered by ACTH (Bird *et al.* 1996). Importantly, co-treatment of the cAMP activator, forskolin, with AII reduced 3β-HSD activity in the same cells (Bird *et al.* 1996). Interestingly, protein kinase C (PKC) activation appears to also inhibit 3β-HSD expression as revealed by addition of staurosporine to the ACTH-treated mouse Y1 adrenal cell line (Reyland 1993). Notably, several studies in both human adrenocortical cells and the H295R cell line have implicated the regulation of 3β-HSD by growth factors such as TGF-β1, EGF and insulin-growth factor (IGF)-II (Doi *et al.* 2001, Feltus *et al.* 2003, Lebrethon *et al.* 1994, Mesiano & Jaffe 1993). Also, dexamethasone treatment has been reported to increase 3β-HSD2 mRNA levels in H295R cells (Feltus *et al.* 2002).

ii) Ovary

The principal isoform expressed in human ovary is 3β-HSD2 (Rheaume *et al.* 1991). The steroidogenic activity of this reproductive organ appears in puberty. Importantly, ovarian steroidogenic capacity in puberty starts at the pre-antral follicle and increases as follicles develop in the pre-ovulatory follicle with a peak steroidogenic capacity and abundant 3β-HSD2 expression in the progestogenic corpus luteum (Dupont *et al.* 1992, Sasano *et al.* 1990). Interestingly, studies have shown an early expression of 3β-HSD in the theca of the fetal ovary. From birth until puberty, low 3β-HSD immunostaining was observed (Dupont *et al.* 1992). From puberty to menopause, the theca interna was the principal cell compartment positive for the enzyme with lower staining in granulosa cells of growing follicles. Induction of the enzyme activity in granulosa cells, mainly in membrana granulosa, was observed at the pre-ovulatory follicle (Sasano *et al.* 1990) and this effect appeared to be mediated by an intracellular increase of cAMP (McAllister *et al.* 1989), adenyl cyclase and protein kinase A (PKA) as a result of the pituitary FSH and LH hormone secretion (Cooke 1999). Interestingly, the absence of 3β-HSD immunoreactivity in theca interna cells overlying the membrana granulosa of the pre-ovulatory follicle suggested that these cells may reflect fibroblast-like cells that are devoid of 3β-HSD activity and are not directly involved in steroidogenesis (Dupont *et al.* 1992, Sasano *et al.* 1990). In post-menopausal ovaries, 3β-HSD was only immunodetected in scattered interstitial cells (Dupont *et al.* 1992). EGF also appeared to positively regulate 3β-HSD as it has been shown in studies with rats (Bendell & Dorrington 1990). *In vitro* studies for the regulation of 3β-HSD in human are very challenging due to limitations of tissue collection at early stages of folliculogenesis. However, more data regarding the intracrinology of the human ovary can be obtained from corpus luteum studies because the collection of lutein cells has been facilitated with *in vitro* fertilisation procedures. Thus, studies with lutein-granulosa cells have shown up-regulation of 3β-HSD by LH, hCG, forskolin and insulin (McAllister *et al.* 1990, McGee *et al.* 1995). Moreover, McAllister *et al.* showed stimulation of cAMP-enhanced 3β-HSD by growth factors such as fibroblast growth factor (FGF), EGF

and TGF-β (McAllister *et al.* 1994). Oestradiol has been demonstrated to antagonise progesterone synthesis, an effect mediated by 3β-HSD abrogation by oestradiol (Fisch & Rose 1994, Vega *et al.* 1994). Finally, luteolytic prostaglandin $PGF_{2\alpha}$ has been found to suppress progesterone synthesis in macaques (Bennegård *et al.* 1991) through rapid down-regulation of 3β-HSD (Duncan *et al.* 1998).

iii) Placenta

The placenta is the organ notable for the production of progestogens and oestrogens during the later stages of pregnancy. The human placenta is positive for the 3β-HSD1 isoform which is mainly located in synciotrophoblastic cells. No evidence of 3β-HSD2 in placenta was reported (Lachance *et al.* 1991). The regulation of the placental enzyme is mainly achieved through activation not only of the PKA pathway that increases intracellular cAMP but also with the PKC pathway as shown with human choriocarcinoma JEG-3 cells (Tremblay & Beaudoin 1993). Importantly, insulin and IGF-I were shown to induce 3β-HSD activity in primary human cytotrophoblasts (Nestler 1989). Also, 3β-HSD1 promoter studies in JEG-3 cells revealed regulation by TEF-5 and a GATA-like factor as noted above (Peng *et al.* 2004).

iv) Breast

Expression of 3β-HSD in mammary gland was first described by Belanger *et al*. who noticed elevated pregnenolone, progesterone, DHEA and androsterone in breast cyst fluid (Bélanger *et al.* 1990). Moreover, the ratio of DHEA and its metabolite androstenedione suggested 3β-HSD activity in the mammary gland. Further studies also showed immunolocalisation of 3β-HSD in breast carcinomas (Sasano *et al.* 1994). Most information about the regulation of 3β-HSD in breast in both health and disease comes from Simard's group who first showed a positive up-regulation of 3β-HSD by IL-4 and IL-13 cytokines along with potential signalling mechanisms that modulate this effect (Gingras *et al.* 1999, 2000). According to these studies, in ZR-75-1 human breast cancer cells, IL-4 signalled through activation of

insulin-receptor substrates (IRS)-1 and IRS-2 which in turn led to transduction of downstream factors such as the PI-3K and MAPK signalling pathways. The inability of IGF-I, insulin or phorbol-12-myristate-13-acetate (PMA) to stimulate 3β-HSD expression in the absence of IL-4 suggested that this response required an IL-4-specific signalling molecule. Regarding this, the STAT-6 transduction signalling pathway was a pre-requisite for IL-4-responsive gene regulation (Shimoda *et al.* 1996, Takeda *et al.* 1996). Thus, the multiple pathways downstream of IRS-1 and IRS-2 could cross-talk with the STAT-6 transcription factor to induce 3β-HSD1 mRNA and protein expression (Gingras *et al.* 2000) (Fig. 1.13).

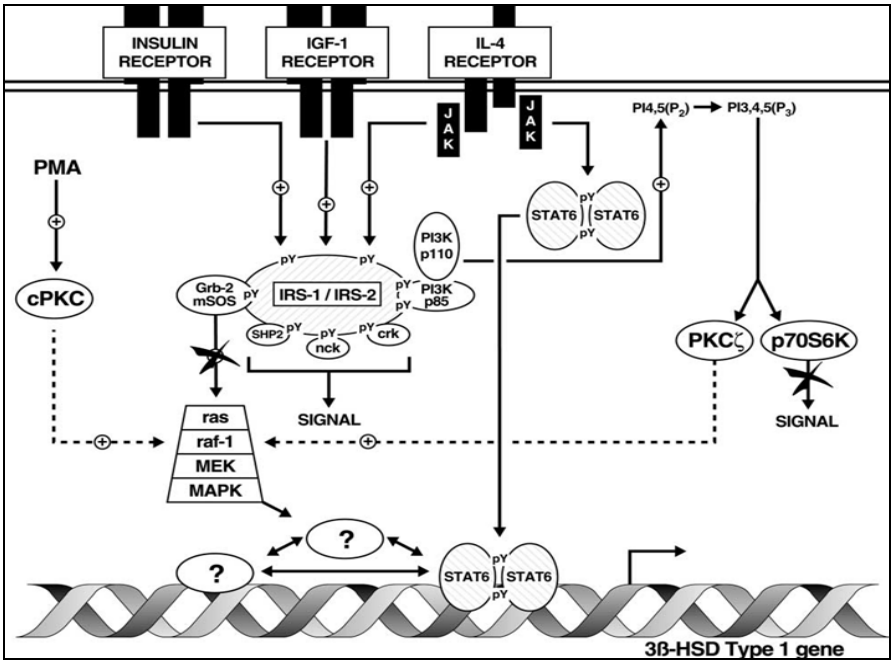

Figure 1.13: IL-4/IL-13 action in ZR-75-1 cell line. This figure summarises the signalling pathways involved in IL-4/IL-13 action. Downloaded from Gingras *et al.* (2001).

1.5 Cytokine Receptor Transactivation and Signalling Networks

As referred to earlier, the two cytokines that have been studied and appear to influence importantly 3β-HSD are firstly IL-1α that *in vitro* suppresses 3β-HSD1 mRNA transcript (Rae *et al.* 2004b) in the human ovarian surface epithelium and also IL-4 that induces 3β-HSD activity in multiple tissues, including breast cancer

cell lines (Gingras *et al.* 1999). These two cytokines exert their effects through binding to cell surface receptors, the IL-1 receptor (IL-1R) and the IL-4 receptor (IL-4R) respectively. Upon their binding to their cognate receptor, transactivation of the latter follows, thereby resulting in the initiation of signalling cascades to mediate cell responses, from cell survival to gene transcription. As stated earlier, IL-1α in the human ovary has been established to be involved in ovulation-associated inflammation and the subsequent injury of hOSE. Regarding IL-4, it has been shown to be secreted mainly at the peri-ovulatory period with a peak at the luteal phase of the menstrual cycle; however, there are no reports of its actions, if any, in the post-ovulatory injury and repair of hOSE. In this regard, IL-1 and IL-4 action in steroid signalling of hOSE at ovulation will be a major focus of this thesis. Therefore, an overview of their action and signalling network will be presented in this section.

1.5.1 The IL-1 system and its signalling networks

1.5.1.1 The IL-1 system: protein interactions and transactivation

The pro-inflammatory cytokine IL-1 has been widely studied due to its pleiotropic role in inflammatory responses in almost every cell type of the body, including the human ovarian cell surface. In human, the IL-1 gene system is located on chromosome 2 and consists of two agonist ligands, IL1-α and IL1-β, two cell surface receptors, the IL-1 type 1 (IL-1R1) and type 2 (IL-1R2) receptors and a receptor antagonist ligand, IL-1-Ra (Webb *et al.* 1986). In human, the two agonist ligands share a 22% similarity and have comparable biological properties, albeit their structure reveals distinct receptor binding sites (Labriola-Tompkins *et al.* 1993, March *et al.* 1985). IL-1R1 and IL-1R2 belong to the toll-like/IL-1 receptor (TIR) superfamily. The feature of these receptors is the presence of the phylogenetically conserved Toll/IL-1R (TIR) cytoplasmic domain that consists of a domain of at least 200aa and is involved in the transduction of innate and inflammatory responses and also contributes to the activation of adaptive immunity, key processes for the restoration of local and systemic inflammatory reactions (Mantovani *et al.* 2007, O'Neill & Dinarello 2000, Rock *et al.* 1998). Receptors of this family can be divided

into two subgroups: those that have three immunoglobulins (Ig) in their extracellular domain, such as the IL-1R, and those whose extracellular segment is composed of leucine-rich repeats, as in the case of toll-like receptors (TLRs). The receptors of this family have been conserved through evolution and appear to mediate signalling pathways associated with pathogen and mechanical injury in insects, plants and mammals.

IL-1α, IL-1β and IL-1Ra responses are exclusively mediated through IL-1R1, the first discovered member of the TIR superfamily, whereas IL-1R2 is a decoy receptor (Sims *et al.* 1988, 1993, Stylianou *et al.* 1992). IL-1R1 is a 80kDa glycoprotein that is composed of a 319aa extracellular domain and a 217aa cytoplasmic domain (Sims *et al.* 1988). After the binding of an agonist ligand, IL-1R1 is phosphorylated by a Ser/Thr kinase, thereby triggering heterodimerisation with another member of the TIR family, the IL-1R accessory protein (IL-1RacP) (Gallis *et al.* 1989, Huang *et al.* 1997). The heterodimer, in turn, interacts with the exclusively cytosolic MyD88 through its TIR domain (Wesche *et al.* 1997). The latter also possesses a death domain that interacts with the death domain of the IL-1-related associated kinase-1 (IRAK-1), a serine-threonine kinase, that is bound to the TNF-receptor-associated-factor-6 (TRAF-6). Intriguingly, it has been demonstrated that IRAK-1 trafficking to the receptor complex is facilitated by IL-1RAcP (Huang *et al.* 1997, Volpe *et al.* 1997). Then, IRAK-4 interacts with the death domain of IRAK-1 resulting in phosphorylation of the latter on Thr 209 and 237 and dissociation of TRAF-6/IRAK-4 complex from MyD88. Following interaction with the TGF-β-activated kinase 1 (TAK-1), IRAKs and TRAF-6 are poly-ubiquitinated and degraded, thereby activating TAK-1 that induces downstream signalling pathways like mitogen-activated protein kinase kinases (MKKs) and inhibitory κB kinases (IKKs) that mediate IL-1-responsive gene transcriptional activity (Dower & Qwarnstrom 2003, O'Neill & Dinarello 2000, Wesche *et al.* 1997, Yamaguchi *et al.* 1995). A simplification of the network involved in IL-1 signalling is illustrated in Figure 1.13.

There are two pathways commonly activated by TIR superfamily members. First, it is the classic inflammatory NF-κB pathway activated by IKK singlasome (Mercurio *et al.* 1997) and secondly it is MAPK pathways, namely the stress/osmotic associated protein kinase/jun N-terminal kinase (SAPK/JNK) and p38 MAPK signalling pathways activated by MKKs, (Freshney *et al.* 1994) (Fig. 1.13). Extracellular signal-regulated kinases 1 and 2 (ERK1/2), albeit mostly activated by mitogenic factors, are also activated by TIR receptors in selective cases (Bird *et al.* 1991, Waterfield *et al.* 2003).

The IL-1R2 is a 60kDa protein and first cloned in 1991 from B cells and expressed in several cell types such as bone marrow cells, neutrophils, keratinocytes and monocytes (McMahan *et al.* 1991). Similarly to IL-1R1, IL-1R2 is encompassed by an extracellular domain, containing three Igs, a transmembrane domain and a cytoplasmic domain of 29aa only, much shorter than the IL-1R1 one, lacking TIR domain. The total homology of the two receptors is only 28%; however, they can both bind the two agonist and antagonist ligands with high affinity, as their extracellular domains share high similarity (McMahan *et al.* 1991). The absence of the TIR domain on the IL-1R2 is probably responsible for the inability of this cell surface receptor to transduce signals, establishing therefore this receptor as a decoy receptor (Colotta *et al.* 1993, Curtis *et al.* 1989, Sims *et al.* 1993, Stylianou *et al.* 1992). Intriguingly, IL-1R2 action is widely considered as a mechanism to counteract inflammatory responses and maintain tissue homeostasis (Mantovani *et al.* 2004, Mantovani *et al.* 2007). For example, it was demonstrated that injection of keratinocytes transfected with human IL-1R2 into mice resulted in attenuated expression of inflammatory cytokines such as IL-6 and it overall decreased the severity of collagen-associated rheumatoid arthritis (Bessis *et al.* 2000). Moreover, transfection of U937 cells (a human leukemic monocyte lymphoma cell line) with IL-1R2 negatively regulated IL-1 secretion (Penton-Rol *et al.* 1997). Further support for the anti-inflammatory effects of IL-1R2 comes from studies with immunosuppressive and anti-inflammatory glucocorticoids and the T helper-2 (Th-2) cytokine, IL-4. Dexamethasone has been reported to enhance IL-1R2 mRNA along

with membrane IL-1R2 binding domain in human polymorphonuclear (PMN) leukocytes (Re *et al.* 1994). Furthermore, IL-4 has been reported to induce IL-1R2 mRNA and most importantly to inhibit IL-1β-induced prolongation of the life-span of PMN cells (Colotta *et al.* 1993). Intriguingly, a proposed mechanism for IL-1R2 negative action is through enhancement of IL-1 ligand affinity for the IL-1R2 by the soluble form of IL-1RAcP (Smith *et al.* 2003).

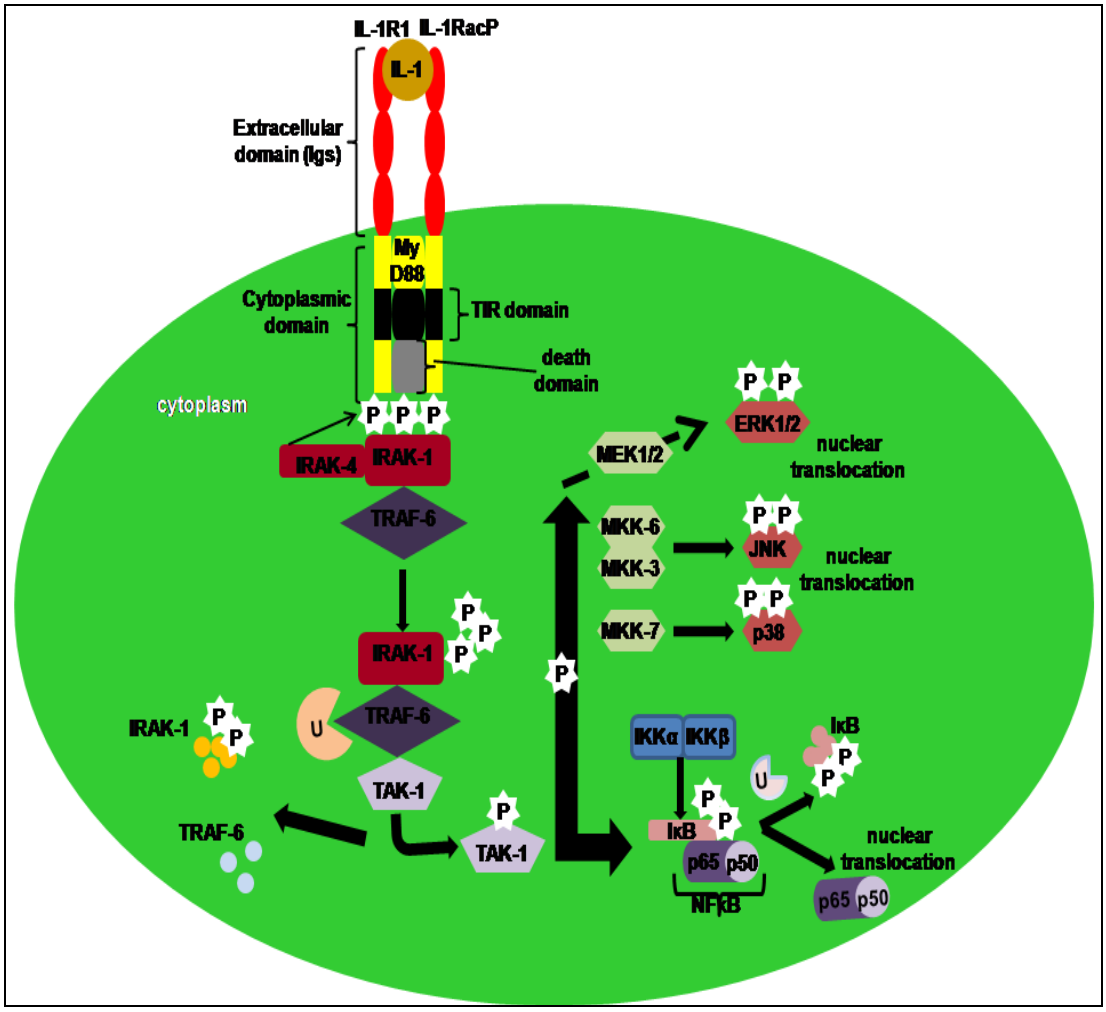

Figure 1.14: The IL-1 signalling network. This cartoon illustrates the main molecules involved in transactivation of IL-1R1 and associated signalling pathways. Binding of IL-1 to the IL-1R heterodimer results in sequential phosphorylation of serine/threonine kinases that in turn mediate transduction of NF-κB and MAPKs molecules.

1.5.1.2 IL-1α-associated signalling transduction pathways

As referred to above, IL-1R1 transactivation leads to activation of signalling pathways that monitor regulation and transcription of responsive genes communally having roles in inflammatory cascades.

i) The NF-κB signalling pathway

NF-κB was first described as a transcription factor that regulated transcription of genes possessing immunoglobulin μ and κ sequences in their promoter (Sen & Baltimore 1986), but it is now realised that it regulates transcription of multiple inflammatory-associated genes. NF-κB is found as a homo- or heterodimer, subunits of which contain a conserved *rel* domain in N-terminus consisting of a dimerisation domain, a nuclear transfer signal domain and a DNA-binding region. It is most commonly encompassed by a 50kDa DNA-binding protein (p50) truncated from its 105kDa precursor (Bours *et al.* 1990) and bound to another DNA-binding 65kDa protein, the p65 subunit (Ghosh *et al.* 1990, Nolan *et al.* 1991). The p50 subunit shares high homology with the proto-oncogene *c-rel* and the *dorsal* gene of *Drosophila*. Similarly, p65 appears to share common characteristics with *rel* gene (Ghosh *et al.* 1990, Kieran *et al.* 1990, Nolan *et al.* 1991). As mentioned above, NF-κB is a downstream activator of the IL-1R1 signalling network. In its inactive form, NF-κB is located in the cytoplasm bound to a member of IκB protein family, all members encompassed by ankyrin-type repeats in their C-terminus, domains that are frequently present in cytoskeleton-associated proteins (Baeuerle & Baltimore 1988a, Baeuerle & Baltimore 1988b). This suggests that anchor cytosolic proteins are probably responsible for association of p50/p65 complex to IκB (Davis *et al.* 1991), thus masking the nuclear transfer signals of the NF-κB that facilitate its import to the nucleus (Beg *et al.* 1992). It has been suggested that the interaction of IκB is achieved through the *rel* domain of the p65 subunit rather than the *rel* domain of the p50 subunit (Nolan *et al.* 1991). Transmission of signals signified by IL-1 binding to its cell surface receptor leads to activation of IKK singlasome (Fig. 1.13) that in turn phosphorylates Ser 32 and Ser 36 of IκB-α, thereby marking IκB for proteolytic

degradation and ubiquitination that allows dissociation and activation of NF-κB (DiDonato *et al.* 1995, Ghosh & Baltimore 1990, Henkel *et al.* 1993, Régnier *et al.* 1997). The release of NF-κB by IκB through a proteolytic-independent tyrosine phosphorylation of the latter has been also proposed (Imbert *et al.* 1996). Additionally, phosphorylation of p65 by PKA has been demonstrated as a pre-requisite for full NF-κB activation before its migration to the nucleus (Zhong *et al.* 1997). As a result, active NF-κB translocates to the nucleus where it can bind to κB DNA sequences of the promoters of target genes and initiate transcription. Inactivation, association and re-translocation of NF-κB/IκB complexes in the cytoplasm are subject to a feedback loop mechanism. Precisely, NF-κB induces transcription of the *MAD*-3 gene that gives rise to the nuclear regulatory protein IκB-α. After its synthesis, nuclear IκB-α interacts with nuclear NF-κB dimers terminating thereby transcriptional activity of the latter in the absence of a new stimulus. The newly-formed IκB/NF-κB complexes migrate to the cytoplasm (Zabel *et al.* 1993).

ii) The MAPK signalling pathways

MAPKs are protein kinases that commonly drive cellular processes, such as gene transcription, proliferation, differentiation, cell survival and cell death (Chang & Karin 2001). They are expressed in all cell types and monitor cellular responses associated with cell surface receptors *e.g* IL-1R1 and IL-4R. Mammals commonly express at least three families of MAPKs, namely ERK1/2 (Boulton *et al.* 1990), SAPK/JNK (Kyriakis *et al.* 1994) and p38 MAPK (Han *et al.* 1994, Rouse *et al.* 1994). Their common characteristic is that they are activated by phosphorylation of the C-terminus at threonine and/or serine residues located laterally adjacent to another residue (x), consistent with the motif Thr-x-Ser. Moreover, all MAPKs possess a proline residue near phosphoacceptor sites probably serving as a recognition marker for kinase activators and inactivators. Activated ERK1/2 is signified by phosphorylation of the $^{202/185}$Thr-Glu-Tyr$^{204/187}$ motif (Butch & Guan 1996, Payne *et al.* 1991), activated SAPK/JNK by phosphorylation of ^{183}Thr-Pro-Tyr^{185} (Hibi *et al.* 1993, Lu *et al.* 1997) and p38 MAPK by phosphorylation of

^{180}Thr-Gly-Tyr^{182} tripeptide (Dérijard *et al.* 1995). The phosphorylation/activation of each MAPK is coordinated by a specific MAPK kinase (MAPKK) such as MEK1/2 for ERK1/2 (Crews *et al.* 1992, Zheng & Guan 1993), MKK3/6 for p38 MAPK (Dérijard *et al.* 1995, Stein *et al.* 1996) and MKK7 for SAPK/JNK (Moriguchi *et al.* 1997). ERK1/2 is predominantly induced by growth factors such as EGF and insulin (Boulton *et al.* 1990, Boulton *et al.* 1991), whilst SAPK/JNK and p38 MAPK are commonly induced by cytokines, ultraviolet (UV) light and osmotic stress (Kyriakis *et al.* 1994, Rouse *et al.* 1994). The action of MAPKs is transient as dephosphorylation by MAPK phosphatases (MKPs) ensues in the absence of a new signal. MKPs can target more than one substrate, however with different efficiency. For example, MKP-1 dephosphorylates p38 MAPK and JNK, thereby blocking UV apoptosis that these MAPKs trigger in U937 leukemic cells (Franklin *et al.* 1998). However, in the same cells, ERK2 inhibition by MPK-1 is less sensitive. Notably, MKP-1 can abrogate the PMA-induced c-Jun and AP-1 transcriptional activity (JNK and JNK/p38 MAPK targets respectively) to a higher degree relative to c-Myc and Elk-1, both transcription factors mediated mainly by ERK1/2. Importantly, MKP-1-dependent inactivation of stress-associated MAPKs appears to be downstream of ERK2 activation, attesting to a cross-talk signalling mechanism among separate MAPKs (Franklin & Kraft 1997). Collectively, it is apparent that the proper communication of each separate MAPK with interacting signalling molecules and substrates is the key for specificity of each member of the MAPK family and prevention of impaired signalling that could be detrimental for the cell fate. In support of this, it has been recently realised that the successful interaction of MAPKs with interacting upstream and downstream molecules is coordinated by amino acid residues adjacent to the catalytic domain of the enzymes, the so-called MAPK-docking sites. Remarkably, all the members of human MAPK family appear to interact with cognate activators, inactivators and substrates through a common domain near the C-terminus of the protein, the common docking (CD) site (Tanoue *et al.* 2000).

Intriguingly, whereas ERK1/2 MAPKs are involved in cell proliferation and survival, p38 MAPK and SAPK/JNK signalling pathways appear to promote pro-apoptotic and cell death procedures. For example, in PC-12 cells (rat adrenal medulla tumour cell line) *in vitro* depletion of neuron growth factor (NGF), a rapid stimulator of mitogenic ERK1/2 (Boulton *et al.* 1991), resulted in activation of p38 MAPK and SAPK/JNK signalling pathways that in turn started apoptotic cascades (Xia *et al.* 1995). Moreover, supplementation with forskolin, insulin or basic fibroblast growth factor, agents that suppress apoptosis through stimulation of ERK1/2 cascades, reversed p38 MAPK and JKN induced activity (Xia *et al.* 1995). Notably, co-transfection of the cells with p38 MAPK and its specific activator MKK3 (Fig. 1.13) in the presence of NGF notably increased p38 MAPK-dependent cell apoptosis (Xia *et al.* 1995). However, despite the similarities of the p38 MAPK and JNK with regard their stimuli and role in cell fate, they significantly differ in upstream (*i.e.* MAPKKs) and downstream molecules (*i.e.* substrates) they interact with (see below), suggesting that these pathways are either complementary or parallel.

The specificity of separate MAPKs is also subject to their responsiveness to distinct stimuli and their activation by separate MAPKKs (see above). Several studies have constistently reported induction of JNK and p38 MAPK by IL-1 in many cell types. In the case of ERK1/2, it is mainly activated by phorbol esters, EGF, insulin and NGF (Boulton *et al.* 1991). For example, in COS cells, IL-1 activated MKK3, but it failed to activate MEK1, the MAPKK of ERK1. Moreover, IL-1 induction of MKK3 increased p38 MAPK activity, an effect that was not reproduced when cells were treated with EGF, a proxy of the MEK-ERK system (Dérijard *et al.* 1995). Interestingly, IL-1-induced MKK3 did not affect JNK activity either, proving that this MAPKK specifically impacts upon p38 MAPK (Dérijard *et al.* 1995). On the other hand, MKK4 was capable of activating both p38 MAPK and JNK (but not ERK1/2), attesting some cross-talk/parallel/common features of stressed-associated pathways (Dérijard *et al.* 1995). However, activation of ERK1/2 by IL-1 has been also reported in a number of cell systems such as HepG2, KB and HGF cells (Bird *et al.* 1991).

Additionally, all three groups of MAPKs target diverse substrates. ERK proteins regulate mitogenic-associated transcription factors such as Elk-1 and c-Myc, whilst p38 MAPK phosphorylates small heat-shock proteins (Freshney *et al.* 1994, Rouse *et al.* 1994) as well as the activating transcription factor-2 (ATF-2) and in some cases AP-1 (Raingeaud *et al.* 1995). Finally, SAPK/JNK activates c-Jun (Dérijard *et al.* 1994, Hibi *et al.* 1993, Kyriakis *et al.* 1994) but also, as p38 MAPK, AP-1 transcriptional activity (Dérijard *et al.* 1994, Moriguchi *et al.* 1997). For example, as expected, in HeLa cells, IL-1-induced p38 MAPK regulated ATF-2, but it did not affect c-Jun or c-Myc (Raingeaud *et al.* 1995).

Numerous studies over the years have been identified the MAPKs that control transcriptional or translational regulation of various genes after treatment with IL-1. To acknowledge some of them, p38 MAPK has been reported to mediate IL-6 and prostaglandin production in human fibroblasts (Funakoshi *et al.* 2001). Moreover, in the same cell type, gelatinase synthesis (ECM component) has been shown to be disrupted upon p38 MAPK inhibition (Funakoshi *et al.* 2001). Furthermore, tissue PA (t-PA) activity appeared to involve ERK1/2 and p38 MAPK pathways in the osteosarcoma U2OS cell line (Chang *et al.* 2006). Also, in odogenic keratocyst fibroblasts, IL-1α-induced COX-2 mRNA appeared to be regulated by all three MAPKs cascades as well as NF-κB pathways (Ogata *et al.* 2007).

Collectively, it is widely accepted that cell signalling transduction pathways are both distinct and common, depending on the end-point and cell type. A common stimulus can result in concomitant signalling cascades not only of all three MAPKs but also of other signalling networks such as the NF-κB signalling pathway for the regulation of a single gene. This effect is not necessarily reproduced in all cell systems. In general, regardless of the specificity that each separate signalling molecule can display, all together they encompass a complex and dynamic network that needs to cooperate accurately to secure cell homeostasis.

1.5.2 The IL-4 system and its signalling networks

1.5.2.1 The IL-4 system: protein interactions and transactivation

IL-4 is a T lymphocyte-associated cytokine produced mainly by Th-2 cells and thus is involved in several cell immune responses with principal role in ameliorating autoimmune and inflammation-associated diseases such as cancer through inhibition of Th-1 responses (Godfrey *et al.* 2000, Nagai & Toi 2000, Nakamura *et al.* 1997, Nelms *et al.* 1999). As mentioned above, IL-4 action is mediated through its coupling to the IL-4R that is expressed in several immune cell types as well as in epithelial cells, including the ovarian cell surface (Burke *et al.* 1996, Lowenthal *et al.* 1988). IL-4R belongs to the family of haematopoietin receptors and consists of two polypeptide chains; the IL-4Rα chain through which signalling transduction is initiated and the γ common (γc) chain that is responsible for the activation of signalling pathways by IL-4Rα (Harada *et al.* 1990, Mosley *et al.* 1989, Russell *et al.* 1993).

IL-4Rα is a 140kDa protein that contains type III fibronectin domains in an extracellular region rich in cysteine residues as well as two tryptophan, serine dipeptides surrounding another amino acid (x) (Trp-Ser-x-Trp-Ser or WSxWS) in the proximal membrane region. The latter is believed to maintain the conformation of the receptor in such a state where it is recognisable by the ligand (Bazan 1990). The IL-4Rα chain has also a cytoplasmic domain that is characterised by a conserved 5-Tyr peptide and a short sequence with serine residues, termed the 'box 1 motif' (Murakami *et al.* 1991, Seldin & Leder 1994). The box 1 motif is located in the proximal membrane region and facilitates interaction with the receptor with Src-related kinases (Minami *et al.* 1993).

IL-4 binding to the IL-4Rα chain results in heterodimerisation with the 70kDa γc chain that is responsible for the activation of downstream signalling cascades by IL-4Rα (Kammer *et al.* 1996, Letzelter *et al.* 1998). Interestingly, neither of the two chains have endogenous kinase activity; this role, however is

fulfilled by janous-family tyrosine kinases (Jak) that attach to cytoplasmic domains of the two chains upon IL-4 binding to the IL-4Rα (Smerz-Bertling & Duschl 1995). Jak-1 is associated with IL-4Rα, whilst Jak-3 with the γc chain (Miyazaki *et al.* 1994, Russell *et al.* 1994, Witthuhn *et al.* 1994). As a result, IL-4Rα is activated through the phosphorylation of the 4 cytoplasmic tyrosines. Site-directed mutations or truncations of the cytoplasmic domain of the IL-4Rα chain have identified that the conserved tyrosine (Y) 497 mediates cell proliferation (Keegan *et al.* 1994), whereas Y575, Y603 and Y631 are responsible for transcriptional regulation of IL-4-responsive genes such as CD23 (Ryan *et al.* 1996). The general concept is that cell proliferation is mediated by the association of IRS1/2 with Y497 after phosphorylation and upon IL-4 binding to the receptor, as revealed in the myeloid cell line 32D (Keegan *et al.* 1994). IRS1/2 can then interact with the PI-3K regulatory subunit or with the Grb-2 adapter molecule initiating thereby PI-3K or ERK1/2 signalling pathways, respectively (Sun *et al.* 1993). Shc is another molecule that can bind to Y497 instead of IRS1/2 and is also capable of transducing ERK1/2 MAPKs (Rozakis-Adcock *et al.* 1992). On the other hand, the attachment of STAT-6 to Y575 and/or Y603 and/or Y631 triggers phosphorylation that in turn induces transcriptional activity of target genes (Reichel *et al.* 1997). Nonetheless, it should be noted that this is an oversimplified concept, as it has been shown that PI-3K can also be involved in the regulation of IL-4-responsive genes and on the other hand STAT-6 could mediate cell survival. For example, the IRS1 pathway has been shown to participate in the regulation of immunoglobulin ε (Wang *et al.* 1997). Moreover, IRS1/2 molecules have been demonstrated to be members of the IL-4-induced machinery that led to stimulation of 3β-HSD1 in human breast cancer cell lines (Gingras *et al.* 2000). Additionally, site-directed mutagenesis of the IL-4Rα chain at residues that function as docking sites for IRS1/2 molecules abolished not only the linkage of IRS1/2 to IL-4Rα but also of STAT-6 activation and induction of the CD23 IL-4-responsive gene (Wang *et al.* 1998). Therefore, the IL-4-related signalling network and responses may differ among cell types and this is part of the complexity hidden behind signalling transduction pathways. A simplification of the IL-4 signalling network is illustrated in Figure 1.14.

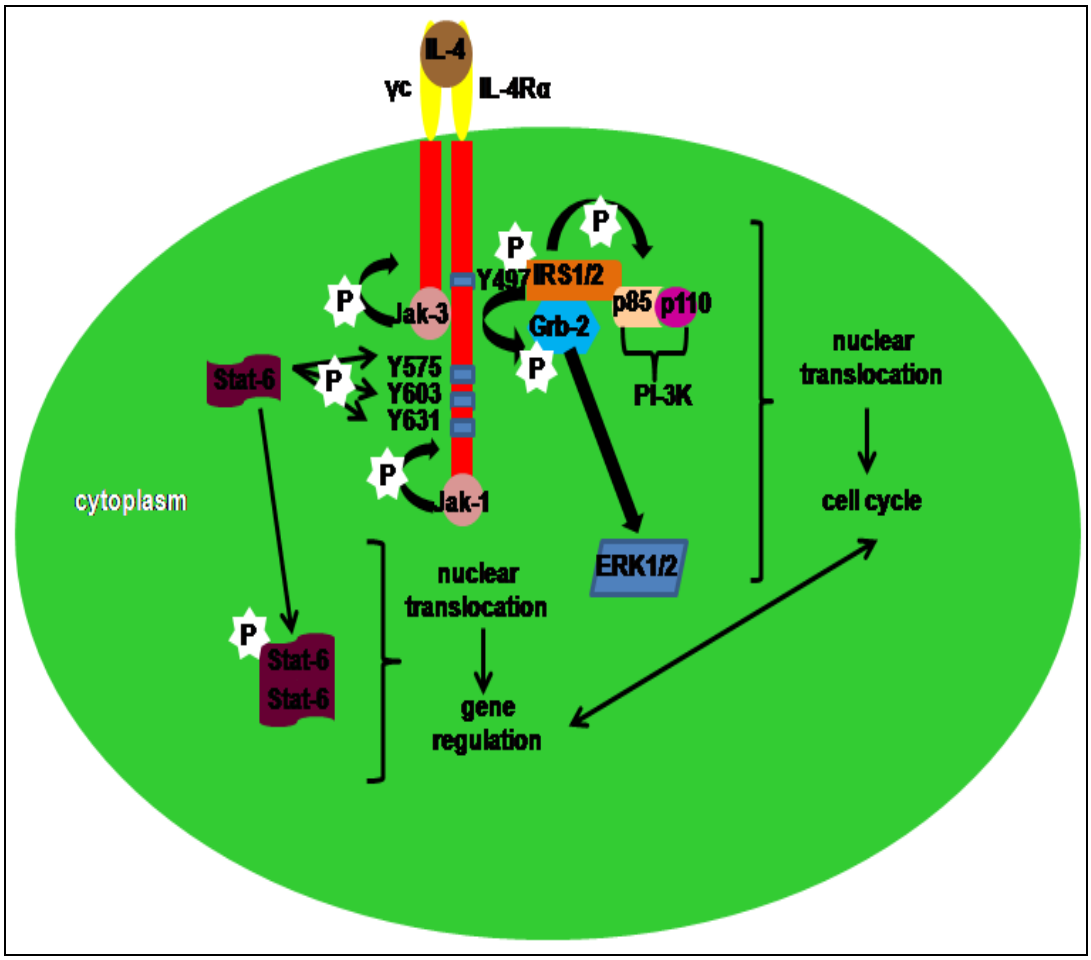

Figure 1.15: The IL-4 signalling network. This cartoon gives an overview of the main molecules that take part in transactivation of IL-4R and associated signalling pathways. Binding of IL-4 in IL-4Rα results in heterodimerisation with γc chain and linkage to tyrosine kinases, Jak-1 and Jak-3 that in turn coordinate sequential phosphorylation of serine/threonine kinases which mediate transduction of IRS1/2 mitogenic pathway and STAT-6 gene regulation pathway. However, participation of IRS1/2 in STAT-6-mediated gene transcription and of STAT-6 in IRS1/2-driven cell proliferation cannot be excluded.

1.5.2.2 IL-4-associated signalling transduction pathways

i) The IRS1/2 signalling pathway

As aforementioned, IRS1/2 phosphorylation is induced by its attachment to Y497 of the IL-4Rα chain and functions as a docking protein for the initiation of signalling cascades that may result either in activation of PI-3K or ERK1/2 kinases or parallel activation of both (Sun *et al.* 1993).

The PI-3K protein is a heterodimer that consists of two subunits; the regulatory p85 (85kDa) subunit (Escobedo *et al.* 1991) and the catalytic p110 (110kDa) subunit (Hiles *et al.* 1992, Otsu *et al.* 1991). Interactions between p85 and p110 subunits are achieved through a 104aa sequence in the C-terminal domain of p85 and a 88aa region of the N-terminal domain of p110 subunit (Dhand *et al.* 1994). In essence, a Tyr-Met-x-Met motif of the phosphorylated IRS1/2 signifies the linkage of p85 subunit to the IRS1/2 and thus it functions as an intermediate that associates tyrosine phosphorylated IRS1/2 with p110 catalytic subunit (Sun *et al.* 1993). Once catalytically active, the p110 subunit is capable of triggering phosphoinositide production that in turn mediates processes such as cell survival and protein synthesis through Ser/Thr phosphorylation of kinases such as PKC and PKB/Akt (Dudek *et al.* 1997, Franke *et al.* 1997).

The ERK1/2 is another downstream activating pathway of the IL-4-induced IRS1/2 (Skolnik *et al.* 1993). In particular, ERK1/2 activation is achieved through the adapter molecule Grb-2 that recognises the phosphorylated Tyr-Val-Ile-Asn motif of IRS1/2 (Sun *et al.* 1993). As such, ERK1/2 MAPKs initiate their mitogenic effects as described above. However, ERK1/2 is not constantly triggered by IL-4. For example, IL-4 does not activate ERK1/2 MAPKs in MC-9, FDC-2 and R6X haematopoietic cell lines (Duronio *et al.* 1992). Also, IL-4-associated cell proliferation are not mediated through activation of ERK1/2 in either FDMAC11 and CT.4S murine myeloid cell lines or primary T cells and mast cells obtained from mice (Welham *et al.* 1994).

An alternative mechanism for the activation of ERK1/2 by IL-4 is through the linkage of the IL-4Rα with Shc, a docking protein that shares similar structural characteristics with IRS1/2 docking proteins (Zhou *et al.* 1995a, Zhou *et al.* 1995b). Precisely, Shc contains one C-terminal and one N-terminal region that can interact with tyrosine-phosphorylated molecules such as Jak-3/IL-4Rα and Grb-2 respectively (Rozakis-Adcock *et al.* 1992, Songyang *et al.* 1993, Zhou *et al.* 1995b). Grb-2 adapter recognises phosphorylated Y317 of Shc and thus initiation of ERK1/2 signalling cascades are fulfilled (Rozakis-Adcock *et al.* 1992).

ii) The STAT-6 signalling pathway

As referred to above, STAT-6 activation by IL-4-transduction of IL-4Rα is implicated in activation of Th-2 responses as well as the transcription of target genes such as CD23, immunoglobulins ε and γ (Reichel *et al.* 1997, Ryan *et al.* 1996, Shimoda *et al.* 1996). STAT-6 interaction with IL-4Rα is achieved by the binding of the N-terminus of one or more STAT-6 molecules to three phosphorylated tyrosines (Y575, Y603, Y631) located in a region between 557 and 657 amino acids of the cytoplasmic domain of IL-4Rα chain (Fig. 1.14) (Wang *et al.* 1996). Upon binding of STAT-6 to IL-4Rα, the former is phosphorylated in the C-terminal domain resulting in its dissociation from the receptor. As such, it triggers homodimerisation with the C-terminus of a second STAT-6 molecule through its docking sites in the N-terminus. Homodimers then translocate to the nucleus where they bind to STAT-6 response elements of target genes (Mikita *et al.* 1996, Schindler *et al.* 1995).

It appears that STAT-6 is a critical component of IL-4 signalling as STAT-6 -/- mice display impaired IL-4 activity. For instance, in STAT-6 deficient mice, IL-4 was unable to induce transcriptional activity of target genes such as CD23 and immunoglobulin ε and also could not promote Th-2 responses (Takeda *et al.* 1996). Remarkably, dysfunction of STAT-6 and not of IL-4R was demonstrated to be responsible for impaired IL-4 signalling (Takeda *et al.* 1996)

1.6 Summary of Chapter 1 and Scope of Thesis

As discussed above, hOSE is a very functional and dynamic tissue that physiologically undergoes cyclical tissue remodelling prior to and post-ovulation. It is capable of secreting and receiving hormones, cytokines and growth factors and its homeostasis is subject to mutually interrelated endocrine, paracrine and intracrine signalling of these molecules.

As detailed, progesterone and progesterone-associated events are protective in the development of ovarian cancer. On the other hand, androgens and androgen-associated situations are considered as risk factors for the initiation of the disease. Moreover, immune mediators appear to impact upon the steroid hormonal milieu of the OSE and inflammatory-associated events such as ovulation promote neoplastic transformation of the ovarian cell surface. The elucidation of the pro-inflammatory and anti-inflammatory steroid mechanisms occurring physiologically in the OSE could improve the poor prognosis of EOC and contribute to the diagnosis and treatment of this fatal disease (Rae & Hillier 2005, Rae *et al.* 2004b). Regarding this, and as mentioned above, from a screen of inflammatory-associated markers in hOSE cells, it was shown that IL-1α suppressed 3β-HSD1 mRNA, one of the 3β-HSD isoforms that drives intracrine generation of progestogens and androgens (Rae *et al.* 2004b). This observation suggested that the 3β-HSD enzyme is under inflammatory control and as such functionality of this enzyme may be critical for hOSE homeostasis. Although, the relevant contribution of 3β-HSD1 and 3β-HSD2 expression to 3β-HSD enzymatic activity is unknown, total 3β-HSD activity in hOSE is expected to determine the availability of progesterone and androgen for signalling via nuclear progesterone (PR) and androgen receptors (AR), respectively. Thus, the scope of this thesis is to investigate the regulation of pre-receptor metabolism and downstream signalling of progesterone and androgens, focusing on 3β-HSD1 and 3β-HSD2 mRNAs, total protein and activity along with the PR and AR in normal hOSE and EOC.

To undertake our studies, we used i) primary hOSE cells that were collected from pre-menopausal women who undertook surgery for benign gynaecological conditions and ii) primary ovarian cancer cells that were obtained from women who were diagnosed with EOC.

Therefore, our central aim can be divided into the following objectives:

a) Investigation of expression pattern of functional 3β-HSD in the human ovarian surface epithelium *in vivo* and *in vitro.*
b) The study of the regulation of 3β-HSD1 and 3β-HSD2 mRNA and total protein and activity by ovarian-associated pro-inflammatory and anti-inflammatory cytokines in primary hOSE cell cultures.
c) The elucidation of transduction signalling pathways through which cytokines affect 3β-HSD1 and 3β-HSD2 transcriptional function in primary hOSE cell cultures.
d) The evaluation of the regulation and transduction of AR and PR mRNA by cytokines that impact upon 3β-HSD1 and/or 3β-HSD2 in primary hOSE cell cultures.
e) Investigation of the functionality of 3β-HSD pathway in primary ovarian cancer cells.

Chapter 2

Subjects and methods

2.1 Tissue Culture

2.1.1 Subjects

2.1.1.1 Physiology (normal ovary)

hOSE cells for *in vitro* experimentation or ovarian tissue for histological studies were obtained from pre-menopausal women who underwent surgery for benign gynaecological disorders, such as fibroids, heavy menstruation and pelvic pain. Subjects who had evidence of endometriosis or receiving any medication that could affect ovarian physiology were excluded from this study as previous reports documented the possibility of lesions in cells collected from endometriotic patients, although they probably might possess good growth potential (Auersperg & Maines-Bandiera 2000). Formal written consent was submitted by all experimental patients and the project was approved by the Lothian Research Ethical Committee (LREC; project numbers 1998/6/33[1], 05/S1103/14[2], 04/S1103/3[3]). Basic clinical information of each patient used for our studies is given in the respective Chapters.

2.1.1.2 Diseased ovary (ovarian cancer)

Cancer material was recruited from patients who were diagnosed with ovarian cancer after diagnostic laparotomy. Solid ovarian tumours and freshly isolated ascites were either plated for *in vitro* culture or were fixed and embedded in paraffin blocks for histological assays. Histological, staging and grading assessment of ovarian cancers was performed by Professor Alistair Williams, Royal Infirmary of Edinburgh (RIE) Department of Pathology. Individual details for each patient are denoted in Chapter 8. Formal written consent was obtained from all patients tested

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[2] Principal Investigator Professor Hilary Critchley.
[3] Principal Investigator Dr Scott Fegan.

and studies were performed according to LREC regulations (project number 04/S1103/44[4]).

2.1.2 Establishment of cell monolayers

Primary hOSE cells and primary ascites were cultured as previously described (Dunfield *et al.* 2002, Hillier *et al.* 1998, Kruk *et al.* 1990). Ovarian surface epithelial cells were scraped from the ovary at an early stage of the surgery to minimise any contamination with blood and stromal cells. 'Flakes' of hOSE cells were then inoculated into culture medium (see below) and transferred to the laboratory. Epithelial cells were grown in donor calf serum (DCS) pre-coated T75 flasks (Corning Inc. Glass Works; Corning NY) containing MCDB 105: M199 media (1:1 v/v), 15% (v/v) fetal bovine serum (FBS), 2mmole/L $_L$-glutamine, 50μg/mL streptomycin and 50IU/mL penicillin (all obtained from Life Technologies, Inc., Renfrewshire, UK, Sigma Chemical Co., Poole, UK and Cambrex, Berkshire, UK) in a humidified tissue culture incubator gassed with 95% air-5% CO_2 at 37^{o}C. Medium was refreshed every seven days. This cell culture protocol allowed cells to reach confluency between 2 and 4 weeks (Hillier *et al.* 1998). A further advantage of 4-week cell culture in FBS-containing medium prior to experimentation was neutralisation of any disparity of different hormonal levels among individual patients that could be encountered as a result of cell collection at different stages of the menstrual cycle (Rae *et al.* 2004a, Yong *et al.* 2002). This step appeared essential, since the use of multiple patients to produce sufficient replicates for each distinct experiment allowed us to overcome restrictions in cell numbers along with limitations in long-term culture of cells from the same subject. Moreover, all the conclusions we make are based on the reproducibility of data obtained from at least three separate patients and therefore are potentially reflective of a broader *in vivo* physiology.

[4] Principal Investigator Dr Scott Fegan.

The purity of epithelial cultures was confirmed with phase-contrast microscopy. Further confirmation that this system gave high purity epithelial cultures was checked in selective cases by staining with a mouse monoclonal antibody that immunoreacts with cytokeratins 5, 6, 8 and 17 or a mouse monoclonal that recognizes cytokeratin 7 (Auersperg *et al.* 2001, Rae *et al.* 2004b, van Niekerk *et al.* 1991). After the establishment of confluent cellular monolayers, the culture medium was removed and cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Sigma) and enzymatically digested by incubation in 5mL trypsin/EDTA (Sigma) in Hank's balance salt solution (HBSS; 0.05% w/v trypsin, 0.5 mM EDTA) at 37^{o}C for 5min. The resultant cell suspension was washed with 10mL DPBS and transferred to universal eppendorf tubes (Sterilin, Birmingham, UK). After centrifugation at 800 g for 3min, the supernatant was discarded. This step was repeated twice after re-suspension of cells in 5mL DPBS. The cells were then re-suspended in 1mL pre-warmed FBS-containing culture medium and the cell number counted using a haemocytometer. Cell viability was assessed used trypan blue (Sigma) staining exclusion criteria. The viability of both primary hOSE cells and ascites ranged between 85 and 95%. Cell suspensions were then taken forward for experimentation.

2.1.3 Experimental treatments

Cell suspensions were adjusted to 3.5x10^{5} viable cells per well (35 mm) of 6-well culture plates for mRNA and protein studies or 10^{5} viable cells per well of 12-well plates (Corning) for 3β-HSD activity assays. After establishment of a cell monolayer (24h), the culture medium was replaced with serum-free medium containing 0.01% bovine serum albumin (BSA) instead of FBS for 24h. Cells were then exposed to the agents of interest (cytokines and/or signalling pathway inhibitors). Incubation times and concentrations applied are denoted in the relevant experiments. In all experimental sets, cells receiving no treatment were used as the control.

2.1.4 Cell harvest

For mRNA studies after experimental treatments, the cell monolayers of 6-well plates were washed twice with 1mL DPBS. Homogenisation of cells was achieved by lysis in 0.35mL guanidine thiocyanate-containing buffer (lysis buffer, RLT; Qiagen, West Sussex, UK) supplemented with 0.01% β-mercaptoethanol (Sigma). The resultant cell lysate was transferred to a 2mL eppendorf tube. To maximise lysis and thus RNA recovery, the lysates were vortexed vigorously for 2min. Lysates were then processed for RNA purification using the RNeasy Mini kit (Qiagen) per manufacturer's guidelines (see below).

For protein assays, hOSE cell monolayers were washed with cold PBS and lysed in 50mM Tris-HCl pH 7.6, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate (all from Sigma) and containing a cocktail of proteinase inhibitors (Roche Diagnostics Gmbh, Manheim, Germany). Lysis was performed on ice and proteins were kept at -80^{o}C until further use.

2.2 Histological Studies

2.2.1 Haematoxylin and eosin staining

Paraffin wax-embedded tissues were cut into 3μm sections and deparaffinised in xylene (2X5min), rehydrated in serial dilutions of ethanol (100, 95, 70 and 50%; 1min each) and washed in PBS (Sigma) and distilled H_2O (2x5min). Then, nuclei were stained in haematoxylin (Sigma) for 20sec followed by removal of excess stain in tap H_2O. Sections were then soaked in eosin for 20sec that specifically stains cytoplasm. Sections were washed well in tap H_2O prior to dehydration in sequential dilutions of ethanol (50, 70, 95 and 100%; 1min each) and cleaning with xylene (2X5min). Slides were mounted with Pertex (Sigma) and when dried were visualised in an Olympus Provis microscope (Olympus Optical, London, UK).

2.2.2 Immunohistochemistry

Immunocytochemistry is an experimental method that allows investigation of the presence or absence of a protein *in situ* in cultured cells or tissue. The development of this approach is based on the antigen-antibody complex that naturally occurs in living organisms. In particular, a primary antibody specifically raised to recognise the target protein (antigen) is applied to the cells or tissue of interest. Then, a secondary antibody against the species in which the primary antibody was raised is added to bind the primary antibody. The secondary antibody is usually labelled with biotin that allows the recognition and amplification of the signal with tertiary antibodies (*i.e.* avidin-biotin complex; ABC) conjugated to horseradish peroxidise (HRP). Finally, a substrate that specifically interacts with HRP is added allowing visualisation (colourimetric reaction) of the signal (Boenisch 2001). Furthermore, the secondary antibody can be labelled with a fluorescent dye, namely Alexa Fluor dyes (usually distributed from Molecular Probes, Poort Gebouw, Netherlands). These dyes fluoresce at different wavelengths (colours) across the spectral range. As such, this method can be routinely used to target more than one protein in the same cell monolayer or tissue section.

2.2.2.1 Colourimetric immunohistochemistry

Sections deparaffinised in xylene, serial dilutions of ethanol, PBS (Sigma, Poole, UK) and distilled H_2O were permeabilised by pressure cooking (20min) or microwaving (15min) in pre-heated 0.01M sodium citrate buffer pH 6.0 (Sigma) followed by cooling for a further 20min under running tap H_2O. In selective cases permeabilisation was not performed. Sequential blocking steps of endogenous peroxidase (3% H_2O_2 in dH_2O (v/v); Sigma) and avidin, biotin (Vector, Peterborough, UK) were then carried out. Each step was followed by PBS washes (2X). Afterwards, slides were submitted to blocking with non-immune normal goat serum (NGS; Sigma) diluted in PBS (1:5 v/v) containing 5% (w/v) BSA for 20min followed by overnight incubation at 4^oC with a rabbit polyclonal antibody raised against recombinant human 3β-HSD2 (Thomas *et al.* 2002b). The dilutions of the 3β-HSD

antibody are denoted in the relevant Chapters. Immunostaining with anti-human mouse monoclonal cytokeratin 7 or anti-human mouse monoclonal cytokeratin 5, 6, 8, 17 (Dako, Ely, Cambridgeshire, UK) diluted 1:1000 in NGS/PBS/BSA at 4^{o}C was also performed. Negative antibody staining controls incubated with matched concentrations of unconjugated rabbit or mouse IgG_1 antibody respectively (Vector, Peterborough, UK) were included routinely. Sequential incubations with anti-rabbit or anti-mouse biotinylated IgG_1 (Vector) and RTU-ABC elite kit (avidin-biotin complex; HRP conjugated; Vector) were each conducted for 1h before removing excess antibody with PBS supplemented with 0.05% Tween 20 (v/v) (PBST; Sigma). Sections were washed three times with PBST between steps. Finally, slides were incubated with HRP-conjugated diaminobenzidine (DAB; Vector) chromagen for 5min and after haematoxylin counterstaining, dehydration in serial ethanol dilutions (50-100%) and xylene was conducted. Antral ovarian follicles served as positive controls for 3β-HSD (Suzuki *et al.* 1993). The immunostained sections were visualized with an Olympus Provis microscope.

2.2.2.2 Fluorescence immunohistochemistry

Cells were passaged into 8-well glass chamber slides (Nunc Lab-Tek; Leicestershire, UK) at a density of 25,000 cells/well and incubated at 37^{o}C/5%CO_2 for 24h. After serum-depletion overnight with medium containing 0.01% BSA (Sigma) instead of FBS, cells were fixed in 100% ice-cold methanol at -20^{o}C for 10min followed by permeabilisation with ice-cold acetone at -20^{o}C for 1min. Sections of ovarian tissues and freshly isolated ascites pellets were processed as above with minor modifications. Incubation with 3β-HSD antibody or unconjugated rabbit IgG_1 antibody (negative control) diluted in 1:400 in NGS/PBS/BSA preceded immunodetection with anti-rabbit IgG_1 antibody directly conjugated to Alexa Fluor 546 (1:200 in PBS; Molecular Probes, Poort Gebouw, The Netherlands), which provides red fluorescent positive staining. After non-immune blocking, incubation with the anti-human mouse monoclonal cytokeratin 7 antibody or the mouse monoclonal cytokeratin 5, 6, 8 and 17 antibody at 4^{o}C overnight (1:300 in

NGS/PBS/BSA) followed by immunodetection with anti-mouse IgG_1 antibody conjugated to Alexa Fluor 488 (1:200 in PBS, Molecular Probes) was performed to yield green fluorescent cytokeratin-positive staining. Slides were counterstained with a nuclear-specific blue fluorescent label, 4,6-diamidino-2-phenylindole (Dapi; Sigma) in 1:1000 dilution in PBS for 10min. Mounting was achieved with Permafluor Aqueous Medium (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Images were captured with a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) at 40X magnification.

Similarly, PR staining was achieved with a commercial mouse anti-human antibody (Novacastra, Newcastle, UK) diluted 1:80. The AR antibody (Santa-Cruz Biotechnology, Heidelberg, Germany) was used at a 1:100 (v/v) dilution and was detected by sequential incubation of biotinylated goat anti-rabbit IgG_1 followed by avidin Alexa Fluor 488 (Molecular Probes).

2.3 Molecular Studies

2.3.1 RNA purification

RNA extraction was performed using the RNeasy Mini kit (Qiagen). This kit allows the purification of RNA longer than 200nt and is based on the selective binding properties of a silica-gel membrane. Addition of 70% ethanol and pure ethanol to the samples and the washing buffer (RPE), respectively, was performed to provide conditions where RNA binds to the silica-gel membrane and the majority of contaminants are washed away (RNeasy Mini Handbook).

After cell harvesting, RNA extraction was performed according to the manufacturer's guidelines (see Appendix 1). The protocol involved the addition of washing buffers (RW and RPE), followed by successive centrifugations. These steps resulted in the separation of the RNA from any contaminants. Finally, RNA was eluted in 30μl RNase-free water. In order to preclude the presence of any DNA which could negatively influence the accuracy of the results in PCR, DNaseI

digestion was carried out using the Qiagen RNase-Free DNase set, on the column during RNA purification. RNA was stored at -80^{o}C until required.

2.3.2 RNA quantity/quality assay

Purified RNA was quantified using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies Inc, Wilmington, DE, USA). Specifically 1.5μL RNA was loaded on to an optical fibre to measure its ultraviolet (UV) absorbance. The RNA concentration was measured at 260nm. To ascertain purity of the RNA, two absorbance ratios were also assessed - the 260/280 and 260/230 ratios to evaluate protein and genomic contamination, respectively. Purified RNA samples were used further only when the ratios were above 2.0.

2.3.3 Semi-quantitative reverse transcription polymerase chain reaction (PCR)

The presence of gene expression (mRNA) of 3β-HSD1, 3β-HSD2, AR and PR mRNA was examined through reverse transcription (RT) and semi-quantitative polymerase chain reaction (sqPCR). GAPDH was also used as a loading control.

DNase-treated RNA was reverse-transcribed to cDNA using the Superscript III kit (Invitrogen, Life Technologies Inc.). A 20μL reaction mixture was prepared as follows:

Reagent	**Final concentration/volume in the reaction mixture**
RT buffer	1 X
$MgCl_2$	5.5mM
oligo dT	0.025μg/mL
0.1M DDT	5mM
dNTP mix	0.5mM
RNase Out	2U/μL
Reverse superscript III transcriptase	10U/μL
2μg RNA+ H_2O	11μL
Final volume	***20μL***

The RNA, oligo dT and dNTP set were incubated at 65^{o}C for 5min (annealing of oligo dT) before the addition of the other reagents. A reaction without reverse transcriptase (RT-ve) for each initial sample was also used as a negative control to ascertain the absence of any genomic contamination. Samples were placed on a thermal cycler (Techne, New Jersey, USA) and a cycle was carried out at 50^{o}C for 50min (reverse transcription) and at 70^{o}C for 15min (deactivation of RT enzyme, denaturation of RNA-cDNA and RNA degradation).

In order to examine the expression of target genes, cDNA was gene-specific PCR-amplified in a 25μL reaction mixture, containing 1X Promega Mastermix (Southampton, UK), 2pmol/mL of the specific forward and reverse primer set, 2μL cDNA and DNase-free H_2O up to 25μL (final concentrations in the reaction mixture). The sequences of each pair of primers and the expected sizes of the amplicons are listed in Table 2.1. A reaction with no reverse transcriptase for each initial sample was used as a negative control (RT-ve). The annealing temperature on the PCR instrument (Techne) was optimised according to the melting temperature (Tm) of each primer based on the formula Tm=2^{o}C (A+C) + 4^{o}C (G+C). Therefore, samples were PCR-amplified as follows:

1 cycle
-Initial Denaturation at 94^{o}C for 3min
37 cycles
-Denaturation at 94^{o}C for 3min
-Annealing: 3β-HSD1 60^{o}C, 3β-HSD2 60^{o}C, AR 60^{o}C, PR 56^{o}C, GAPDH 60^{o}C for 30sec
-Extension at 72^{o}C for 1min
1 cycle
- Final Extension at 72^{o}C for 5min

PCR amplicons were electrophoresed in a 1.5% agarose gel at 100V and lanes were visualised with 0.03μg/μL (final concentration in the agarose gel mixture) ethidium bromide (Sigma) staining under UV light and were assessed with a 100bp ladder (Blueskript SK+:Sau3A1; in-house).

Table 2.1 Sequences of sqPCR primers

Gene	FW Primer (5'-3')	RV Primer (5'-3')	Fragment size (bp)	Annealing Tm ^{o}C	NCBI accession number
3β-HSD1	TCATCCG CCTCTTG GTG	CTACCTCT ATGCTACT GGTGTAG	339	60	NM_000862
3β-HSD2	TGGTCCGC CTGTTGGT GGAA	CTACCTCT ATGCTACT GGTGTAG	329	60	NM_000198.2
AR	ATTGTCCA TCTTGTCG TCTTCG	CACACTAC ACCTGGCT CAATGG	228	60	NM_000044.2; NM_001011645.1
PR	GATTCAG AAGCCAG CCAGAG	TGCCACAT GGTAAGG CATAA	766	56	NM_000926
GAPDH	ACCACAG TCCATGCC ATCAC	TCCACCAC CCTGTTGC TGTA	431	60	BC_083511.1

FW: forward, RV: reverse, bp: base pairs, Tm: melting temperature

2.3.4 Quantitative reverse transcription polymerase chain reaction (Taqman)

Two-step Taqman quantitative PCR (qPCR) was performed to measure the transcription levels of human 3β-HSD1, 3β-HSD2, IL-1R1, IL-4R, AR, PR, LOX and COX-2 mRNA.

Using a first strand cDNA synthesis kit (AB Applied Biosystems, Applera, UK), DNase-treated RNA was reverse transcribed to cDNA in a 10μL reaction mixture as follows:

Reagent	**Final concentration in the reaction mixture**
RT buffer	1X
$MgCl_2$	5.5mM
Random hexamers	2.5μM
RNase inhibitors	0.4U/μL
dNTP mix	200μM
Taqman reverse transcriptase	1.25U/μL
200ng RNA	40-50ng
RNase-free H_2O	Up to 10μL
Final volume	***10μL***

Oligo dT primer was not added, since in qPCR, 18S, which does not have a poly-A tail, was used as an internal reference. A reaction without reverse transcriptase (RT-ve) and a reaction with RNase-free H_2O instead of RNA were also run to preclude potential genomic or reagent contaminations, respectively (negative controls). One cycle was carried out at 25^{o}C for 1h (annealing of random hexamers), one cycle at 48^{o}C for 45min (reverse transcription) and one at 95^{o}C for 5min (deactivation of RT enzyme, denaturation of RNA-cDNA double strand and RNA degradation).

Following cDNA synthesis, transcription levels were assessed through Taqman Real-Time qPCR. qPCR is a sensitive and reproducible method that detects the amount of the product that is amplified relative to the amount of the initial template in each cycle of the PCR. The major advantage of this technique is the measurement of the kinetics of the reaction in the early phases of the PCR-amplification (exponential-growth phase) and the detection of even a two-fold change (two copies of the product) (Applied Biosystems 2005). In this regard, the different amounts of each product at any given cycle number and not at an end-point can be determined.

Real-Time PCR is based on the detection and quantification of a fluorescent signal emitted by a fluorescent reporter incorporated in a hydrolysis probe as a result of the 5'-exonuclease activity of the Taq polymerase (see below). There are three current forms of hydrolysis probes; i) molecular beacons, ii) scorpions and iii) Taqman probes (Dorak 2004). In our methodology, Taqman probes were used as fluorescent reporters.

The Taqman probes are 20-30bp oligonucleotides and are complementary to a sequence surrounded by the forward and reverse primers of the target genes. These probes are labelled with the fluorescent dye FAM on the 5'- end and the quenching dye TAMRA or MGB on the 3'- end. In each reaction, when the probe is intact, energy is transferred from its 5'- fluorescent molecule to its 3'- quenching molecule, a procedure known as FRET (fluorescence resonance energy transfer). In contrast, when the probe is bound on the replicating template, Taq polymerase cleaves the 5'-end of the probe due to its 5'- exonuclease activity. Therefore, FRET ceases and fluorescence starts to be emitted proportionally to the probe cleavage (Fig. 2.1) (Applied Biosystems 2005, Bustin 2002, Bustin & Nolan 2004, Dorak 2004, Ginzinger 2002).

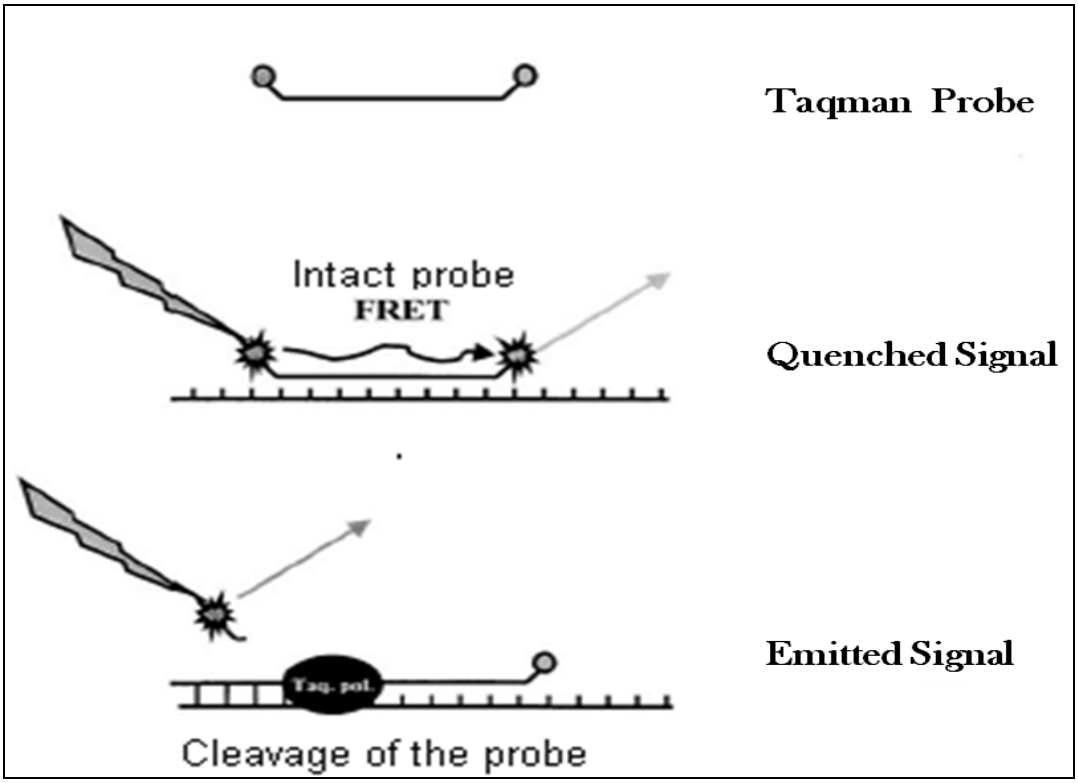


Figure 2.1: Function of Taqman probes. The probe anneals to a sequence of the target gene between the forward and reverse primers. FRET is generated between the 5'- and 3'- end of the intact probe, until the polymerase cleaves the 5'- end of the probe while it adds bases to the growing strand of DNA. Therefore, fluorescence is released proportional to the probe cleavage. Taken from Ginzinger (2002).

For the optimization of the results, the ribosomal RNA 18S was used as an internal control. The 18S probe was labeled with VIC dye, since FAM and VIC have the largest difference in emission maximum (Dorak 2004).

cDNA (2µL) was used for qPCR, using commercial Applied Biosystems reagents. Each reaction was carried out in duplicate. The primers were pre-validated (Assay-On-Demand systems, Applied Biosystems) or were designed using the Primer-Express software (Perkin-Elmer, Beaconsfield, Bucks, UK). Details of the

primers/probes sets are listed in Table 2.3. Each reaction was run in a 25μL mixture containing the following (final concentrations):

- 1X of the universal 2X Taqman mastermix

  -PCR buffer 10X
  -5.5mM $MgCl_2$
  -200μM dNTPs (400μM dUTP)
  -1.25U/μL AmpliTaq DNA Polymerase,
  -Uracil-N-glycosylase (UNG)

- 50nmol/L 18S forward and reverse primers and probe mixture
- 20X of the assay-on-demand primers and probe mixture of the target genes (AB Applied Biosystems)
- nuclease-free H_2O up to 23μL

A RT-ve, a RT-H_2O control (both come from reverse transcription) and a non-template control (NTC, 2μl nuclease-free H_2O instead of cDNA) were used as negative controls in each run (Applied Biosystems 2005, Dorak 2004).

Taqman reactions were carried out in an ABI Prism 7900 Sequence Detector (AB Applied Biosystems, Warrington, UK) following the default protocol (Dorak 2004, Applied Biosystems Manual):

1 cycle
- 50 ^oC for 2min for the activation of UNG [UNG prevents re-amplification of carry-over PCR products by removing any uracil incorporated into amplicons (Dorak, 2004)

1cycle
- 95^oC for 10min (Taq Polymerase activation)

40 cycles
- Denaturation at 95^oC for 15sec
- Annealing and extension at 60^oC for 1min

Data displayed in linear and logarithmic plots were analysed using the ABI Prism 7900 Sequence Detector Software. More precisely, the cycle at which the fluorescent signal of the sample (point at which the amplicon starts to be accumulated; threshold cycle (C_T)) was sufficiently greater (>10standard deviations) than the baseline fluorescence (fluorescent signal that is below the limit of detection of the instrument) was assessed. Since 18S amplification started very early in each run, the baseline for 18S was adjusted between 3 and 8 cycles (start and stop baseline values respectively), whereas the baseline of the target genes was, by default, from cycles 3-15. The C_T was always adjusted within the exponential-growth phase above any baseline value (Fig. 2.2) and according to the C_T of the internal normaliser (18S). This assessment also reflected the cycle at which each separate sample was significantly PCR-amplified above the baseline (C_T value for each sample). Therefore, the C_T was set in such a way that replicate C_T values were as close as possible (Applied Biosystems 2005, Bustin 2002, Bustin & Nolan 2004, Dorak 2004, Ginzinger 2002). After amplification plot analysis, results were exported to Excel software.

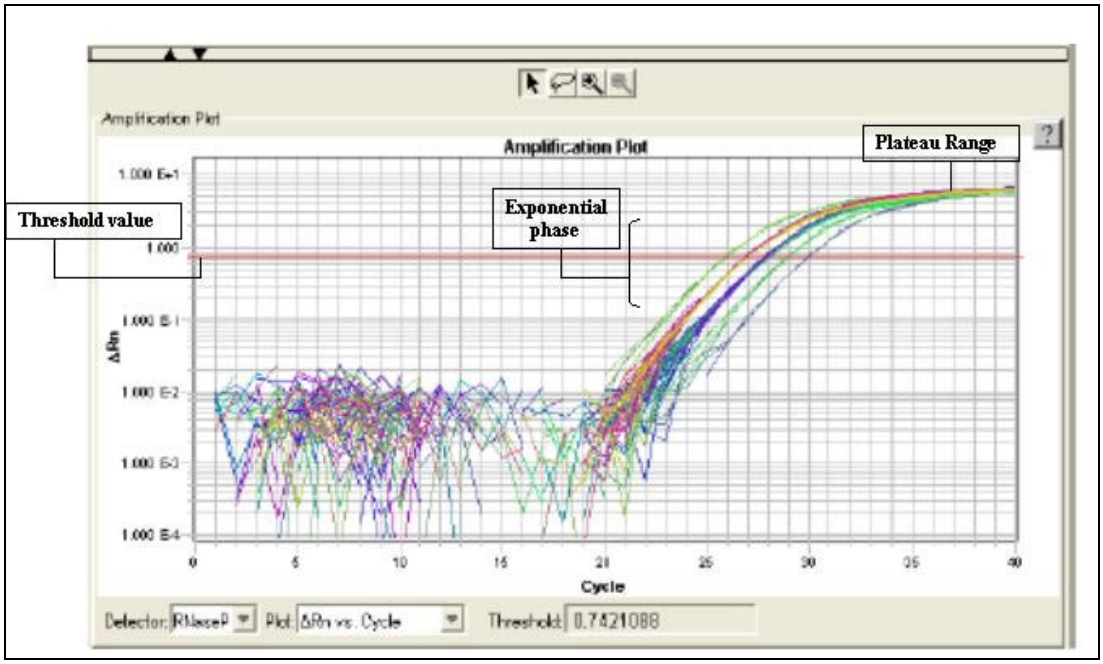


Figure 2.2: Logarithmic amplification plot of a Taqman qPCR run. Detection of the amplification of each sample at the exponential-growth phase and not at an end point (plateau range). The critical parameters for an efficient analysis are illustrated in the figure. Threshold cycle has been set up within the exponential growth phase of the reaction, in a way where C_T values of replicates are tight. Baseline has been adjusted from 3 to 15 cycles (default). Taken from http://docs.appliedbiosystems.com/pebiodocs/04364014.pdf. (2005).

The Ct values were used to calculate the abundance of the mRNA of each sample. Firstly, the difference between the C_T of the target and the internal control was calculated (dC_T). As each sample was run in duplicate, the mean dC_T was ascertained. Then, the mean dC_T of the control (sample with no treatments) was subtracted from the mean dCt of each sample (ddC_T). Finally, the relative copy number between the control and each sample was determined by the formula $2^{-(ddC_T)}$.

Table 2.2 Sequences of primer/probes sets for qPCR

Gene	FW Primer (5'-3')	RV Primer (5'-3')	Probe (5'FAM-TAMRA/MGB 3')	NCBI accession number
3β-HSD1	Assay-on-demand	Assay-on-demand	Assay-on-demand	NM_000862
3β-HSD2	Assay-on-demand	Assay-on-demand	Assay-on-demand	NM_000198.2
IL-1R1	TGTCACCG GCCAGTTG AGT	GCACTGGG TCATCTTCA TCAATT	ACATTGCTTACT GGAAGTGGAAT GGGTCAG	NM_000877
IL-4R	Assay-on-demand	Assay-on-demand	Assay-on-demand	X_52425
AR	GTACCCTG GCGGCAT GGT	CCCATTTCG CTTTTGACA CA	AGCAGAGTGCC CTATCCCAGTCC CA	NM_000044
PR	CAGTGGG CGTTCCAA ATGA	TGGTGGAA TCAACTGTA TGCTTGA	AGCCAAGC CCTAAGCCAGA GATTCACTTT	NM_000926
LOX	Assay-on-demand	Assay-on-demand	Assay-on-demand	NM_002317.3
COX-2	CCTTCCTC CTGTGCC TGATG	ACAATCCA TTTGAACA GGAAGCT	TGCCCGACTCC CTTGGGTGTCA	U_04636

FW: forward, RV: reverse

2.4 Protein and Activity Studies

2.4.1 Protein quantification

After homogenisation of cells in protein lysis buffer as described in section 2.1.4, cell extracts were prepared by sonication for 20sec. A sample of protein (20μL) was removed, diluted in distilled H_2O 1:50 and quantified with the Bradford assay (Bradford 1976) in duplicate using a Cobas Fara analyser (Roche Diagnostics Gmbh, Manheim, Germany). Quantification was carried out after the addition of Coomassie Brilliant Blue solution (100mg Coomassie Brilliant Blue G-250 in 50mL 95% ethanol added to 100mL 85% (w/v) phosphoric acid) to the protein samples followed by measurement of the absorbance at 595nm. This protein evaluation method is based on the finding that the binding of an acidic solution of Coomassie Brilliant Blue to a protein is accompanied by a change in absorbance from 465nm to 595nm. As such, interactions (hydrophobic and ionic) stabilise the anionic form of the dye, producing a visible colour change (Bradford 1976, http://www.ruf.rice.edu/~bioslabs/methods/protein/bradford.html (2008)).

2.4.2 Western immunoblotting

Total protein (25μg/μL) was size-fractionated by electrophoresis (12% SDS/PAGE) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA) followed by blocking in 5% dried semi-skimmed milk diluted in PBS containing 0.05% Tween 20 (PBST) for 2h. An overnight incubation at 4^oC with anti-human rabbit 3β-HSD (1:20,000) or with anti-human rabbit AR (1:1000) or anti-human rabbit actin (1:1000; Abcam, Cambridge, UK) diluted in 1% dried semi-skimmed milk/PBST was followed by 1h-incubation with anti-rabbit IgG_1 conjugated to HRP (Sigma) in 1:10,000 in 1% milk/PBST. Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL) detection kit (Millipore). Human placenta (Thomas *et al.* 1989, Thomas *et al.* 1988) and human H295 adrenocortical cells (Rainey *et al.* 1994) were used to ascertain that the 3β-HSD antibody detected both

human isoforms. The breast cancer T47D cell line was used as positive control for AR (Buchanan *et al.* 2005, Chalbos *et al.* 1987).

2.4.3 3β-HSD activity assay

After treatments cell monolayers were prepared for 3β-HSD enzymatic assay with minor modifications to that previously described (Gingras *et al.* 1999). Treatment media were removed and 1mL serum-free medium containing 0.5μM pregnenolone (Preg) and 150,000cpm/μL ^{3}[H]-Preg were added to initiate a reaction reflecting 3β-HSD dehydrogenase/isomerase activity. Culture medium with no cells inoculated with Preg and ^{3}[H]-Preg as well as Prog and ^{3}[H]-Prog were also included and served as standards. After incubation at 37^{o}C for 8h, the medium was transferred to a 12mL glass centrifuge tube and mixed well with dichloromethane (8mL) to extract steroids into the organic phase. Then, samples were centrifuged at 2,000rpm for 15min, the aqueous phase was removed and the organic phase (containing the steroids) was concentrated under a nitrogen stream. After reconstitution in 100μL dichloromethane and vigorous vortexing, samples were applied to silica-gel pre-coated sheets (PE, SIL G; Whatman, Maidstan, Kent, UK) and developed for 1.5h in a tank containing 92% chloroform/8% ethanol as the mobile phase (150mL). Finally, the TLC sheet was scanned using a Bioscan 2000 radioactive imaging detector (Lablogic Systems, Sheffield, UK). Radio-labelled steroids were separated according to their mobility on the TLC plate. Identification of the mobility of each steroid on the silica-gel pre-coated sheet was facilitated with concomitant loading of the standards (see above). Radioactivity peaks were measured as percentage (%) of the substrate conversion to the active steroid and data were computed as pmoles of substrate conversion per hour (pmoles/h).

2.5 Statistical Analysis

All data from each experimental set were combined and presented as means and standard errors (±sem). The numbers of replicate experiments are denoted by the number (n) of independent replicates and are given in figure legends and text. Basic

statistical analysis was performed, using one-way analysis of variance (ANOVA) with the GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, USA). Repeated measures ANOVA and Newman-Keuls post-hoc testing were run for multiple comparisons, whereas two-tailed paired Student t-tests were performed for single comparisons. Regarding Taqman results, in order to avoid any potential bias yielded by transformations through the equation 2^{-ddCt} for the optimisation of the relative copy number of the target genes related to untreated control, all statistics were performed at the dCt level (raw data), thus avoiding a situation of no variability about the mean of control groups, and permitting correct interpretation of statistical values obtained. A p value <0.05 was taken as statistically significant in robust data sets.

Chapter 3

Establishment of hOSE cell culture system: Expression of 3β-HSD in the human ovarian surface epithelium

3.1 Introduction

3.1.1 Collection and culture of human OSE cells

Protocols for the successful collection and culture of human OSE have been mainly developed by Nelly Auersperg's group. First efforts to culture human OSE were first described in 1984 (Auersperg *et al.* 1984), further established in 1988 (Siemens & Auersperg 1988) and simplified and improved in 1990 (Kruk *et al.* 1990). Problems encountered at culturing OSE cells in plastic plates involved transformation of cells to a fibroblast-like phenotype with time in culture due to the use of inappropriate nutrient media and/or supplementation of growth factors such as EGF and cortisol (Auersperg *et al.* 1984, Siemens & Auersperg 1988). However, the method described by Kruk *et al.* (Kruk *et al.* 1990) appeared the most efficient one and closer to the *in vivo* biology and behaviour of the human OSE. This method involved a gentle scrape of the ovarian surface with a sterile scraper at the time of surgery and direct placement of cell scrapings in sterile culture medium. This cultural system yielded essentially pure epithelial cell monolayers with minimal or no stromal contamination. The growth potential of individual cell cultures was not affected by diagnosis, patient age and phase of the menstrual cycle when surgery took place (Kruk *et al.* 1990). Also, there were not apparent phenotype changings even after up to four subcultures. Inability of cell monolayers to reach confluency within a month outlined senescence (Kruk *et al.* 1990).

Notably, it was demonstrated that OSE cells in culture resembled regenerative OSE cells that heal peri- and post-ovulatory after injury rather than stationary OSE cells of an anovulatory cycle. Cultured hOSE cells have been shown to undertake epithelio-mesenchymal transitions, especially when explanted in three-dimensional culture (collagen-coated plates, Matrigel), thus mimicking a physiologic *in vivo* post-ovulatory response (Kruk *et al.* 1994). Indeed, hOSE cell monolayers have been proved to express both epithelial and stromal markers (Dyck *et al.* 1996). On the other hand, OSE cells recruited from patients with a strong family history of ovarian cancer (FH-OSE) have been demonstrated to be more committed to an

epithelial phenotype, a feature of epithelial ovarian cancer, than the OSE of patients with no family history for ovarian cancer (NFH-OSE) (Dyck *et al.* 1996).

3.1.2 Markers of *in vitro* culture of the OSE

OSE is a multipotent mesothelium that is not committed to an epithelial phenotype. Regarding this, cultured OSE cells are positive for epithelial markers such as collagen IV and laminin along with fibroblast markers such as vimentin and collagen types I and III. Intriguingly, whereas collagen type III is present in cultured hOSE cells, it is absent in stationary hOSE cells *in vivo* and this phenotype agrees with enhanced collagen III secretion in post-ovulatory healing when normal OSE cells transit to a more mesenchymal-fibroblastic phenotype (Dyck *et al.* 1996). Cytokeratins 5, 6, 7, 8 and 17 are also present both *in situ* and *in vitro* but they are progressively lost within time in culture when cells take on a more fibroblastic-like phenotype (Auersperg & Maines-Bandiera, Dyck *et al.* 1996), a feature that is not encountered in immortalised OSE cell lines, FH-OSE and EOC cells (Auersperg *et al.* 1994a, van Niekerk *et al.* 1991). Moreover, E-cadherin and CA125, molecules that are highly expressed in epithelial ovarian cancers, are not secreted by cultured OSE cells or immortalised cell lines, reflecting their non-malignant character (Auersperg *et al.* 1994a, Auersperg *et al.* 1999). Finally, OSE cells have 17β-HSD activity, an invaluable marker to distinguish between OSE and mesothelial cells of extraovarian origin (Auersperg *et al.* 1984, Blaustein 1984).

3.1.3 3β-HSD expression in different cellular compartments of the human ovary

As highlighted in Chapter 1, the importance of 3β-HSD is subject to the fact that the synthesis of all classic steroid hormones is downstream of 3β-HSD action. In human, the adrenal is the major source of inactive substrates formed by cholesterol such as Preg, DHEA-S and DHEA that are subsequently converted into active products *in situ* or are transferred to target tissues through the circulation to be catalysed into more active components. The expression pattern, localisation and regulation of 3β-HSD in the human ovary were described in detail in Chapter 1

(1.4.2.3ii). Briefly, 3β-HSD2 is the principal isoform expressed in human ovarian steroidogenic cells with a peak expression in the luteal phase of the menstrual cycle. Low amounts of 3β-HSD1 has been reported to be expressed in the corpus luteum as well (Rheaume *et al.* 1991).

To date, there have been no previous studies on functional 3β-HSD expression in the human ovarian surface epithelium, the cellular compartment that is believed to be responsible for >85% of ovarian cancers. A microarray analysis showed the presence of 3β-HSD1 mRNA in hOSE and its regulation by IL-1α, though the biological significance of this response is still obscure (Rae *et al.* 2004b). Interestingly, immunohistochemical studies in rat OSE showed an absence of 3β-HSD (Adams & Auersperg 1981). As discussed in Chapter 1 (Section 1.4.2), 3β-HSD is the enzyme which catalyses the final reaction for the intracrine generation of active progesterone, the steroid hormone which is considered to protect from ovarian cancer (Risch 1998). Therefore, the potential *de novo* capacity of hOSE for local regeneration of progesterone and its downstream signalling could be an additive mechanism for hOSE itself to protect OSE cells from genetic damage. Moreover, novel aspects of regulation of ovarian steroid biosynthesis in health and disease might be revealed.

3.1.4 Aim

The first aim of this chapter is to present the hOSE system we used to study the regulation of steroid signalling in the human ovarian surface epithelium *in vivo* and *in vitro*. Following optimisation of cell cultural conditions based on previous protocols (Hillier *et al.* 1998, Kruk *et al.* 1990), we propose to investigate the potential expression of 3β-HSD in the hOSE cell layer *in situ* and also in an *in vitro* cell culture system.

3.2 Subjects and Methods

Assessment of the expression of 3β-HSD protein and activity along with the isoform expression pattern was performed using immunohistochemistry (colourimetric (IHC) and immunofluorescence (IF) methods), radiometric activity assays and PCR respectively. Immunohistochemistry assays involved the use of whole ovarian tissue (*in situ* expression) as well as immunostaining of cultured hOSE cell monolayers (*in vitro*). Also, immunohistochemical studies for cytokeratins (CK) were performed to identify whether CK staining overlapped with 3β-HSD expression. For an *in vivo* approach we used human ovaries from pre-menopausal women who underwent ovariectomy due to non-malignant gynaecological conditions, whereas 4-week cultured hOSE samples from 3 separate patients were used for the *in vitro* approach. Representative pictures are depicted herein. Samples from five other patients were used to measure basal 3β-HSD activity. Investigation of the isoform expression pattern, 3β-HSD1 and 3β-HSD2 mRNA, was performed using semi-quantitative (sq) and quantitative (q) RT-PCR (Taqman).The clinical profile of the individual patients used for these studies is presented in Tables 3.1 and 3.2.

Table 3.1 *In situ* studies

Patient No	Age (yrs)	Reason for surgery	Phase of menstrual cycle	IHC	IF
1	29	Amenorrhoeic past 5yrs; cyclical pain	-	3β-HSD, CK	3β-HSD, CK
2	40	Dysmenorrhoea; constant bleeding	-	3β-HSD, CK	3β-HSD, CK
3	40	Constant bleeding for 7 months; endometriosis history	-	3β-HSD, CK	3β-HSD, CK

IHC: immunohistochemistry, IF: immunofluorescence

Table 3.2 *In vitro* studies

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
4	5433	04/S1103/36	39	TAH	Cyclical pain	(5) Follicular	TLC
5	5447	04/S1103/36	42	TAH	Fibroids	(19) Luteal	TLC
6	5484	05/S1103/14	32	TAH	Prolapse	(24) Luteal	TLC
7	5497	04/S1103/36	39	TAH	Pelvic pain; dysmenorrhoea	n/s	TLC
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	TLC
9	7229	04/S1103/36	40	TAH	HMB	(2) Follicular	TLC
10	5536	04/S1103/36	23	DiagLapar	HMB/pain	n/s	sqPCR
11	5537	04/S1103/36	24	DiagLapar	Dysmenorrhoea	(28) Luteal	sqPCR
12	7383	04/S1103/36	43	TAHBSO	Fibroids	n/s	sqPCR
13	7384	04/S1103/36	42	TAH	HMB	(23) Luteal	sqPCR
14	9014	04/S1103/36	32	DiagLapar	Pelvic pain	n/s	sqPCR
15	5410	05/S1103/14	36	LAVH	HMB; Dysmenorrhoea	(17) Luteal	qPCR
4	5433	04/S1103/36	39	TAH	Cyclical pain	(5) Follicular	qPCR
16	5434	1998/6/33	41	TAHBSO	Fibroids	(3) Follicular	qPCR
17	5441	05/S1103/14	44	TAH	HMB	(25) Luteal	qPCR
18	7246	04/S1103/36	46	TAHBSO	Fibroids	(16) Luteal	qPCR
19	7249	04/S1103/36	44	STAH	Dysmenorrhoea	(8) Follicular	qPCR
14	9014	04/S1103/36	32	DiagLapar	Pain	n/s	qPCR
10	5536	04/S1103/36	23	DiagLapar	HMB/pain	n/s	IF
20	7423	04/S1103/36	49	TAH	Fibroids	(30) menstruation	IF

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, STAH: sub-total abdominal hysterectomy, LAVH: laparoscopic assisted vaginal hysterectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. TLC: thin layer chromatography, IF: immunofluorescence

3.3 Results

3.3.1 Morphology of the human ovary and human ovarian surface epithelium

3.3.1.1 Haematoxylin and eosin staining of human ovary

Prior to immunostaining, ovaries were subjected to haematoxylin and eosin (H+E) staining to identify the structure of the different cell compartments and populations. Fig. 3.1 depicts the morphology of the various ovarian compartments of a representative human pre-menopausal ovary. As described in previous sections, the hOSE is a single cellular layer that surrounds the ovary and consists of heterogenous epithelial cells (Fig. 3.1 a,d,e). Representative pictures of columnar (d) and rounded (e) epithelial cells are illustrated. Intriguingly, some sloughed OSE is also depicted (d), showing that hOSE can easily detach from the surface. A well-structured corpus luteum, the most steroidogenic structure of the female body, is also illustrated (b). The corpus luteum consists of acute steroidogenic cells with big nuclei (lutein-granulosa), as well as small cells (lutein-theca) (c) and stromal cells such as fibroblast-like and immune cells. Finally, an antral follicle is also shown (f) that consists of several layers of granulosa cells that expand between the cumulus oophorus (not shown here) and the membrana granulosa (f). The membrana granulosa layer is surrounded by a basal lamina that separates granulosa cells from theca layer that is differentiated into theca interna and theca externa (f).

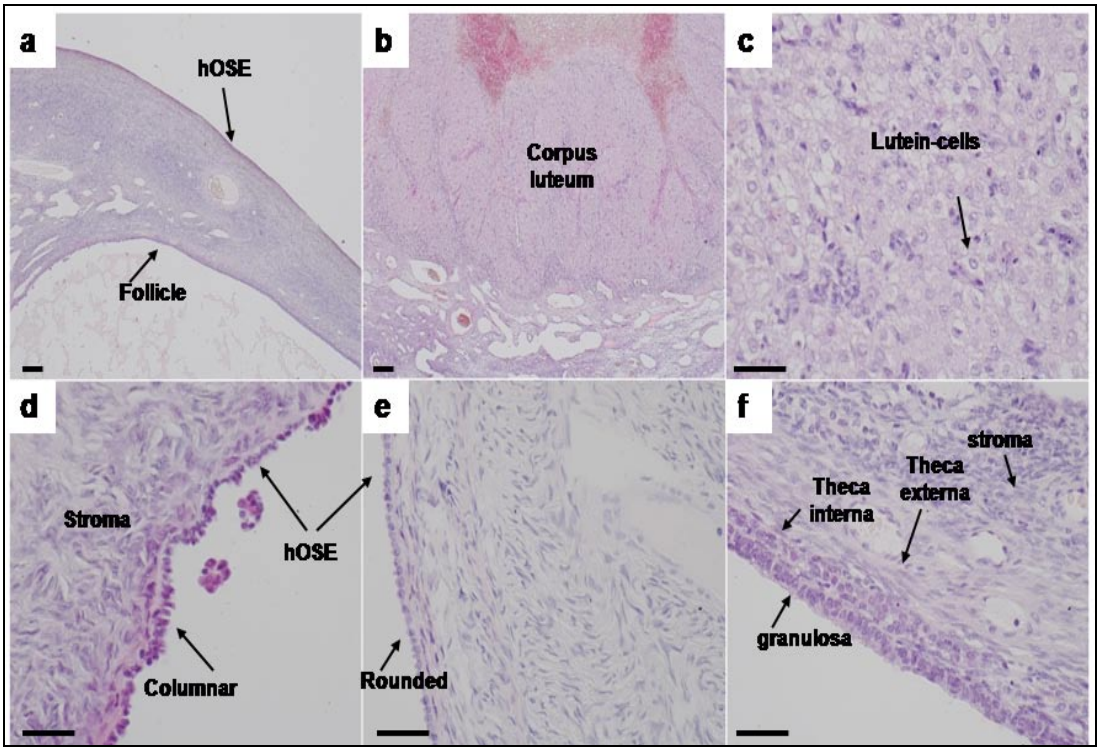


Figure 3.1: Haematoxylin and eosin staining of a human ovary. a) hOSE layer surrounds the ovary and is separated from the stroma by a basal lamina. A mature follicle (antrum) is also demonstrated, b) a well-structured corpus luteum, the most vasculature and steroidogenic structure of the female body, c) lutein-cells of the corpus luteum, d), e) hOSE cells; notice that this layer consists of cuboidal cells that differ in shape and size (columnar and rounded at this slide), f) a closer approach of a big follicle. Granulosa cells are enveloped by a basal lamina that separates them from theca interna and theca externa. Slides were visualised in Olympus Provis microscope and pictures were captured with a digital D30 Canon camera. Scale bars: a, b) 200μm, c-f) 50μm.

3.3.1.2 Morphology of hOSE cell cultures

As described in Chapter 2, the surface of the ovary was scraped and flakes of hOSE cells were transferred into DCS-precoated T75 culture flasks for *in vitro* manipulation. Cells were cultured until confluency (2-4 weeks) and were either processed for further experimentation and/or passed to another flask (passage 1) for later use. Figure 3.2 illustrates hOSE cell morphology at different stages of culture. On day 0, hOSE flakes float in culture medium (a). Cell attachment is achieved between day 1 and 5 (not shown) and hOSE colonies start to form until they cover the surface of the flask, forming a 'cobblestone' monolayer (b, c). As seen in (b) and (c), cells have a squamous-to-cuboidal shape, typical of their epithelial phenotype and are kept tightly the one to the other. Cells subcultured for another 1 week do not differ morphologically from the primary (passage 0) cultures (d, e). In some cases, stromal contaminations were observed to interrupt epithelial colonies and fibroblast-like cells appeared to predominate over epithelial cells (f). Those cell cultures were discarded and not processed for experimentation. Bright field phase-contrast microscopy appeared essential to monitor stromal contamination that could negatively affect confidence of data obtained in subsequent assays. In selective cases, epithelial purity was ascertained with epithelial-specific marker staining (see below).

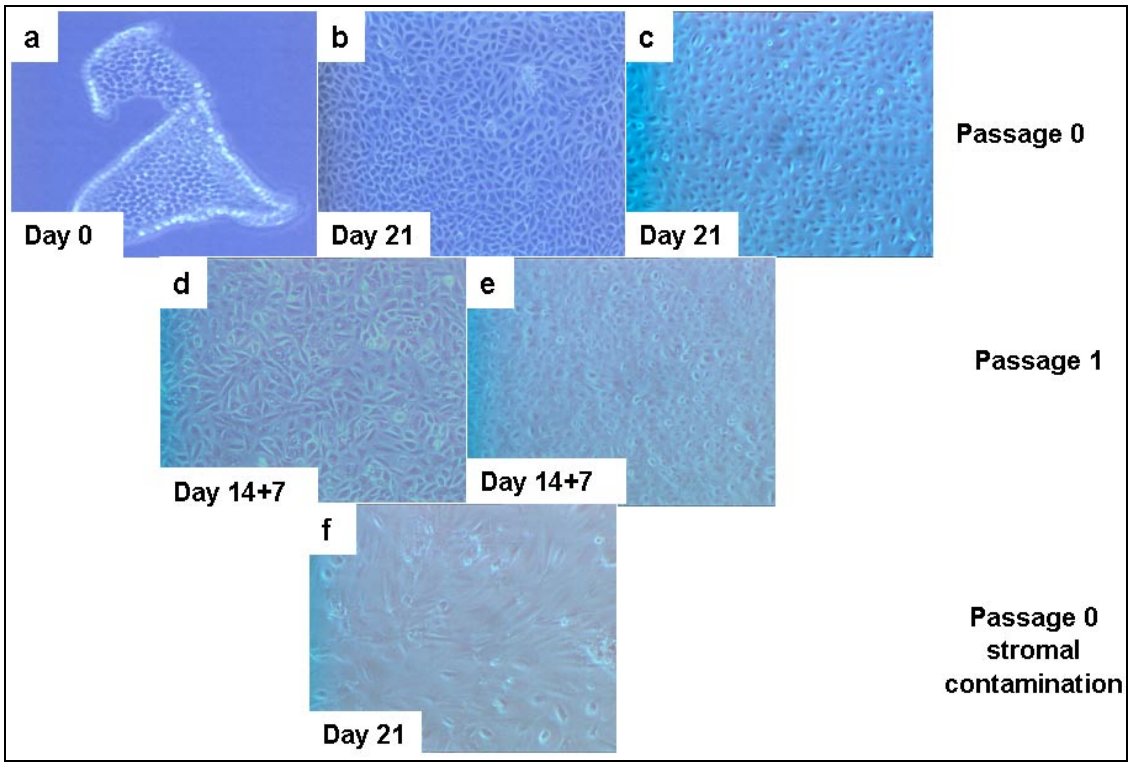


Figure 3.2: Morphology of cultured hOSE cell monolayers. a) hOSE cell scrapings on the day of collection, b, c) a confluent hOSE cell monolayer after 21 days in culture (pictures captured with different lenses of inverted microscope), d, e) hOSE cell monolayers in subculture, f) hOSE cell culture that is contaminated with fibroblast-like cells. Pictures were captured with a Zeiss phase contrast microscope, 5X magnification (scale bars for the camera used are not provided).

3.3.2 Characterisation of hOSE cells by immunostaining with epithelial-specific markers

Recognition of epithelial cells of the human ovary was achieved through immunostaining with epithelial-specific markers (Fig. 3.3). Cytokeratin 7 (left column) and pancytokeratin 5, 6, 8, 17 (centre column) antibodies (both in 1:1000 dilution) stained the OSE layer of the human ovary. As expected, neither antibody stained ovarian granulosa (3^{rd} slide left and centre columns). Unconjugated mouse IGg_1 as negatives did not react with DAB chromagen, showing that production of the brown staining came from specific antibody-antigen interaction. Slides were visualised with an Olympus Provis microscope and pictures captured with the attached 30D Canon digital camera.

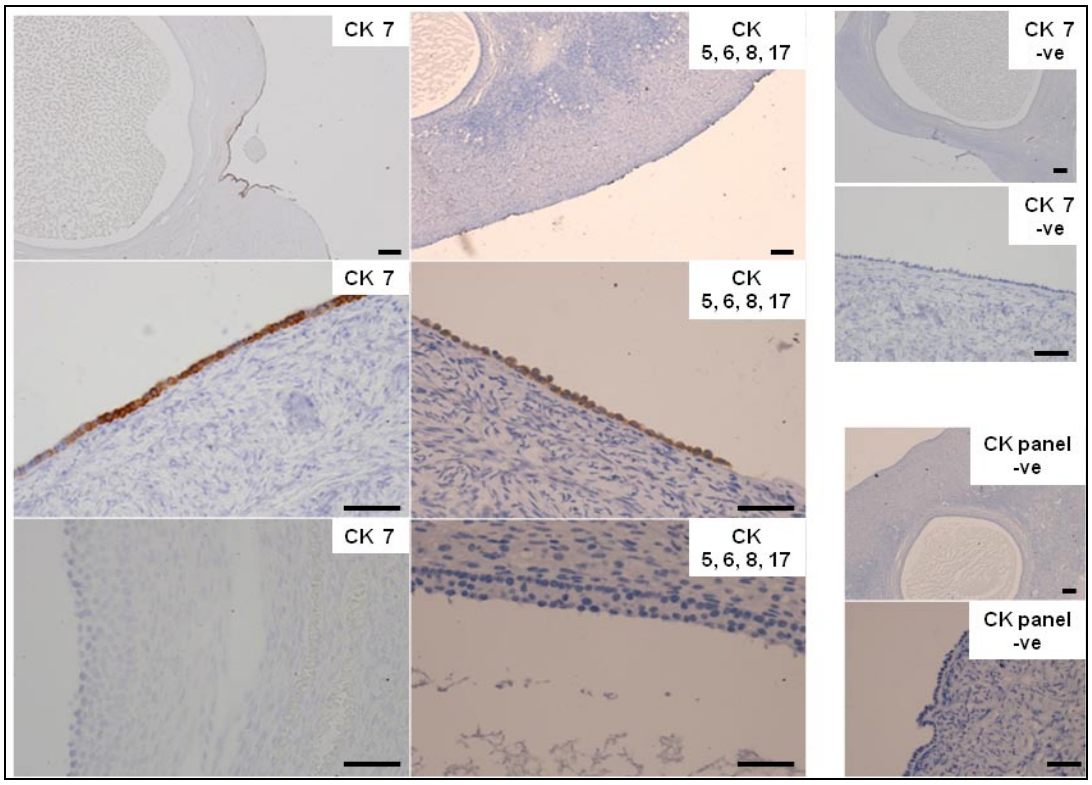


Figure 3.3: Immunostaining of a human ovary for cytokeratins (CK). Cytokeratin staining was performed with two different mouse monoclonal antibodies. The left column shows staining for CK 7. Granulosa cells were used as negative controls (left column, 3rd slide). Centre column shows staining with the CK panel (5, 6, 8, 17). Granulosa again served as the negative control (centre column, 3rd slide). The right panel shows negative controls for both antibodies. Pictures were captured with an Olympus Provis microscope in 4X and 40X magnifications. Scale bars 50µm and 200µm.

3.3.3 Expression of 3β-HSD in the human ovarian surface epithelium

As described above, 3β-HSD is present in ovarian follicular cells with a peak expression in the corpus luteum. Investigation of the potential expression of 3β-HSD in the hOSE monolayer required strict optimisation of the antibody, because antibody dilutions suitable for granulosa and corpus luteum staining might not be optimal for hOSE cells if expression levels were greatly different; inversely, strong immunostaining of hOSE cells could yield background elsewhere in the ovary. In this section, immunostaining with different protocols are presented. Only satisfactory antibody dilutions are demonstrated.

3.3.3.1 Colourimetric immunolocalisation of 3β-HSD in the hOSE layer of the human ovary (DAB staining)

i) No antigen retrieval treatment

Immunostaining of human ovaries with 3β-HSD antibody in serial dilutions (1:500, 1:1000, 1:2000) without an antigen retrieval step (Fig. 3.4 A) showed intense staining of human granulosa cells of a pre-ovulatory follicle (c,d) but staining in hOSE layer was very low at the 1:1000 dilution (a) and undetectable at a 1:2000 dilution (b). Data with 1:500 are not shown, since there was unspecific background throughout the whole tissue. Profound cytoplasmic staining in corpus luteum was also observed (Fig. 3.4 Da).

ii) Pressure cooker treatment

Another approach used for 3β-HSD immunolocalisation was unmasking the epitopes through pressure cooking. Serial antibody dilutions (1:1000, 1:2000, 1:4000 and 1:6000) showed notable immunostaining, however staining was expanded into nuclei as well as stromal cells even at highest dilutions (1:6000, Fig 3.4 B). Corpus luteum staining was cytoplasmic and specific to steroidogenic cells (Fig. 3.4 Db).

iii) Microwave treatment

A revelation of 3β-HSD loci in human ovarian tissue was also achieved using a microwave heating step. Serial dilutions of the 3β-HSD antibody (1:1000, 1:2000, 1:4000 and 1:6000) showed specific and cytoplasmic staining in the granulosa and theca interna (1:6000; Fig. 3.4 Ca-c) as well as in hOSE cells (d-f) with minimal or no unspecific staining of stroma (d-f). Cytoplasmic-specific staining is illustrated more clearly in slides Fig. 3C (j,k). Importantly, the staining intensity differed among OSE cells of different shape. Columnar (d) and rounded (e) (cells A) OSE cell staining appeared stronger relative to flat OSE (cells B) (f) cells (Gillett *et al.* 1991, Papadaki & Beilby 1971). The 3β-HSD antibody specifically stained steroidogenic cells of the corpus luteum (Fig. 3.4 Dc). Negative slides with unconjugated IgG_1 antibodies at matched concentrations were run routinely with all the different protocols. Only representative slides are shown here (Fig. 3.4 C g-i).

Figure 3.4 A

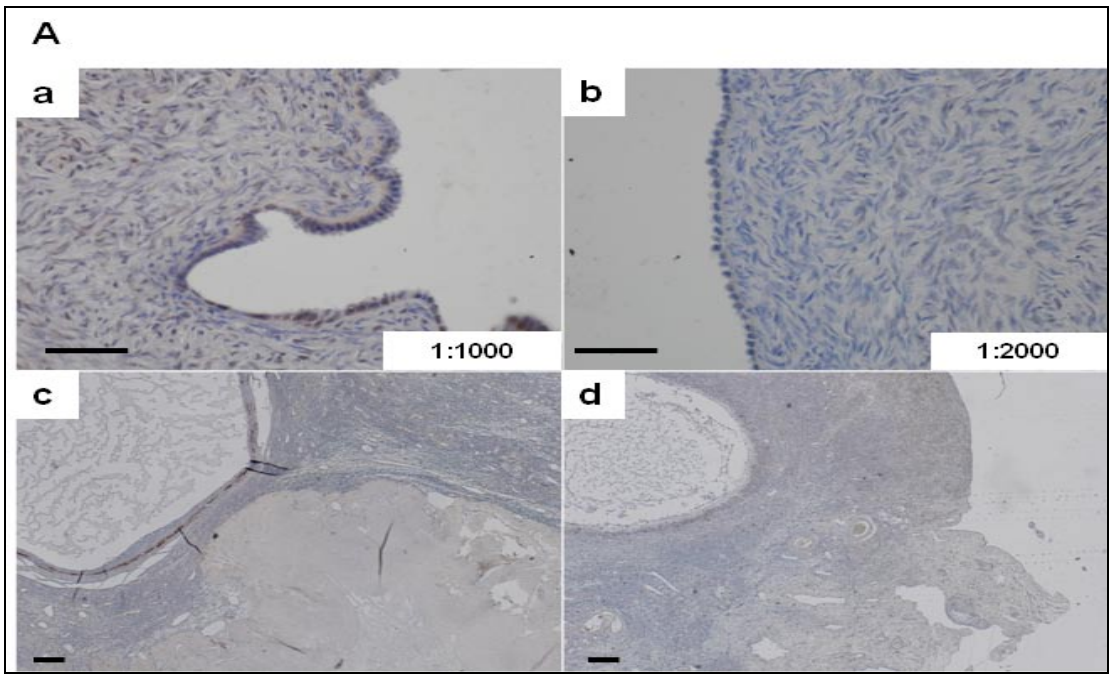


Figure 3.4 B

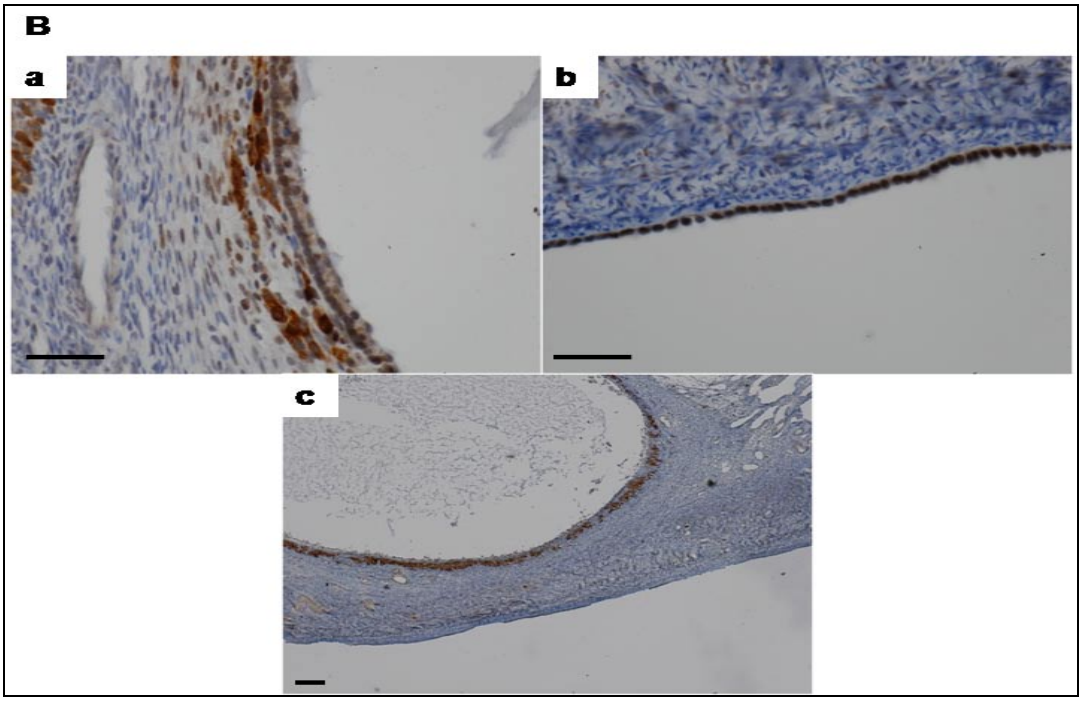
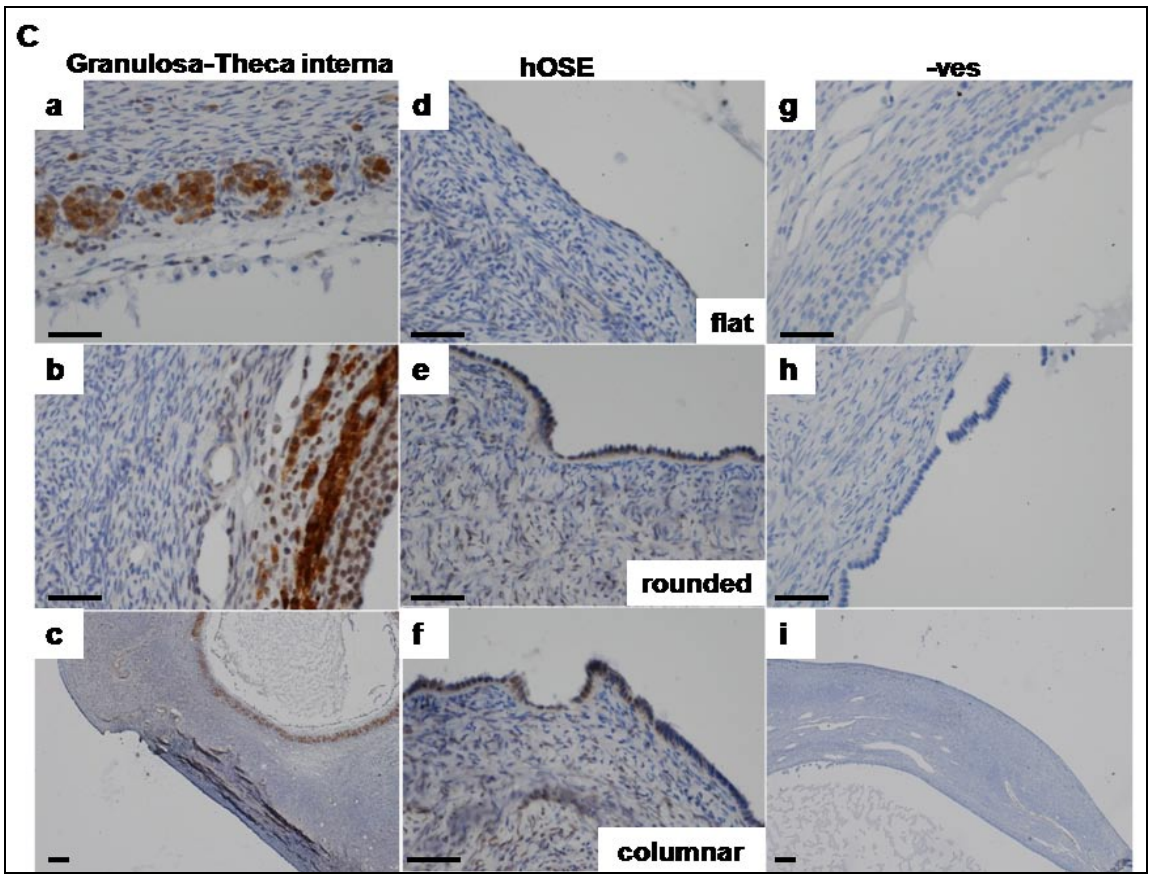


Figure 3.4 C


C
Granulosa-Theca interna
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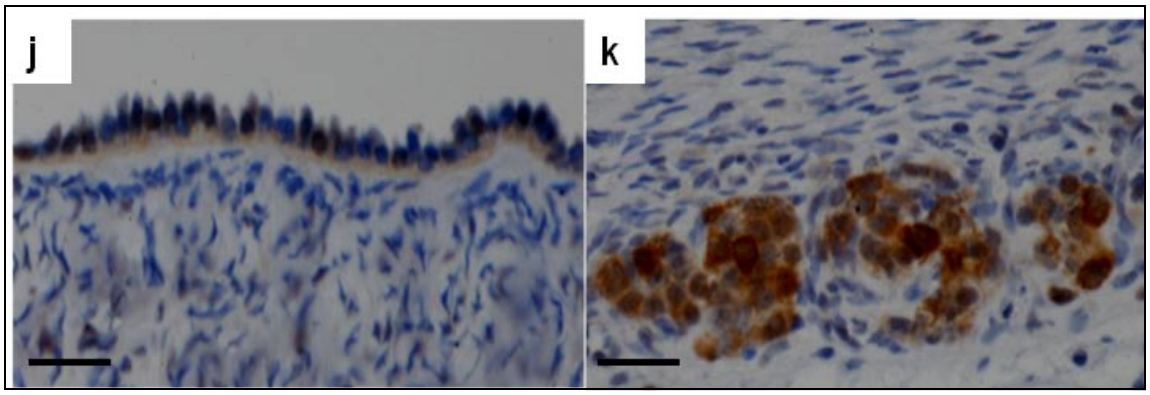
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Figure 3.4 D

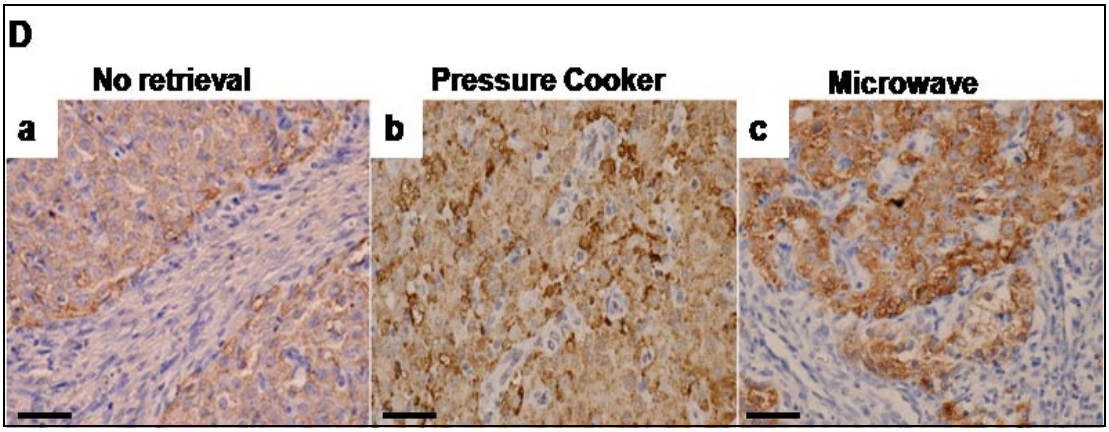


Figure 3.4 (p 111-113): 3β-HSD immunostaining of the human ovary. A) Immunostaining without antigen retrieval yielded the optimal staining in human granulosa (c,d), though low or no detection of 3β-HSD was observed in hOSE (a,b; 1:1000, 1:2000). B) Antigen retrieval with pressure cooker treatment (1:6000 dilution of 3β-HSD antibody) strongly stained hOSE cells, although, staining was expanded to nuclei of both hOSE and granulosa cells (a,b,c). C) Antigen retrieval with microwave heating system and the use of 1:6000 dilution of the antibody. Cytoplasmic staining was observed in both follicular (a-c) and hOSE (d-f) cells with different intensity among different cell types of OSE (d-f). Cytoplasmic staining is more obvious in j and k pictures that reflect part of the (a) and (e) ones (cropped). Negatives were not stained after DAB exposure (g-i). D) Staining of the corpus luteum with all three protocols. Cytoplasmic staining was observed in all three cases. As expected, the antibody stained specifically steroidogenic cells, with no staining in stroma (a, b, c). Scale bars: A, B (a,b), C (a,b,d-h,j,k) and D (a,b,c) 50μm, A (c,d), B (c), C (c,i) 200μm.

3.3.3.2 Co-localisation of 3β-HSD with epithelial-specific cytokeratins in hOSE layer

Further confirmation of epithelial-specific immunostainig of 3β-HSD was performed through indirect double immunofluorescence and confocal microscopy (Fig 3.5). Ovarian tissue was submitted to serial staining with 3β-HSD antibody (1:400 dilution; red fluorescence) and two different cytokeratin antibodies (1:300 dilution; green fluorescence); a mouse monoclonal that reacts with 5, 6, 8, 17 cytokeratins (a-d) and a mouse monoclonal antibody that binds to cytokeratin 7 (e-h). Confocal microscopy revealed cytoplasmic staining of cytokeratins and 3β-HSD as expected with no staining in stroma. Granulosa cells and theca interna of a big follicle served as controls for antibody staining specificity (i-l). They appeared positive for 3β-HSD and negative for cytokeratins (only granulosa stained with CK7 are shown here). Counterstaining with Dapi was performed to visualise cell nucleus (a, e, i). Pictures were captured with Zeiss LSM 510 Meta Axiovert 100M confocal microscope at 40X magnification.

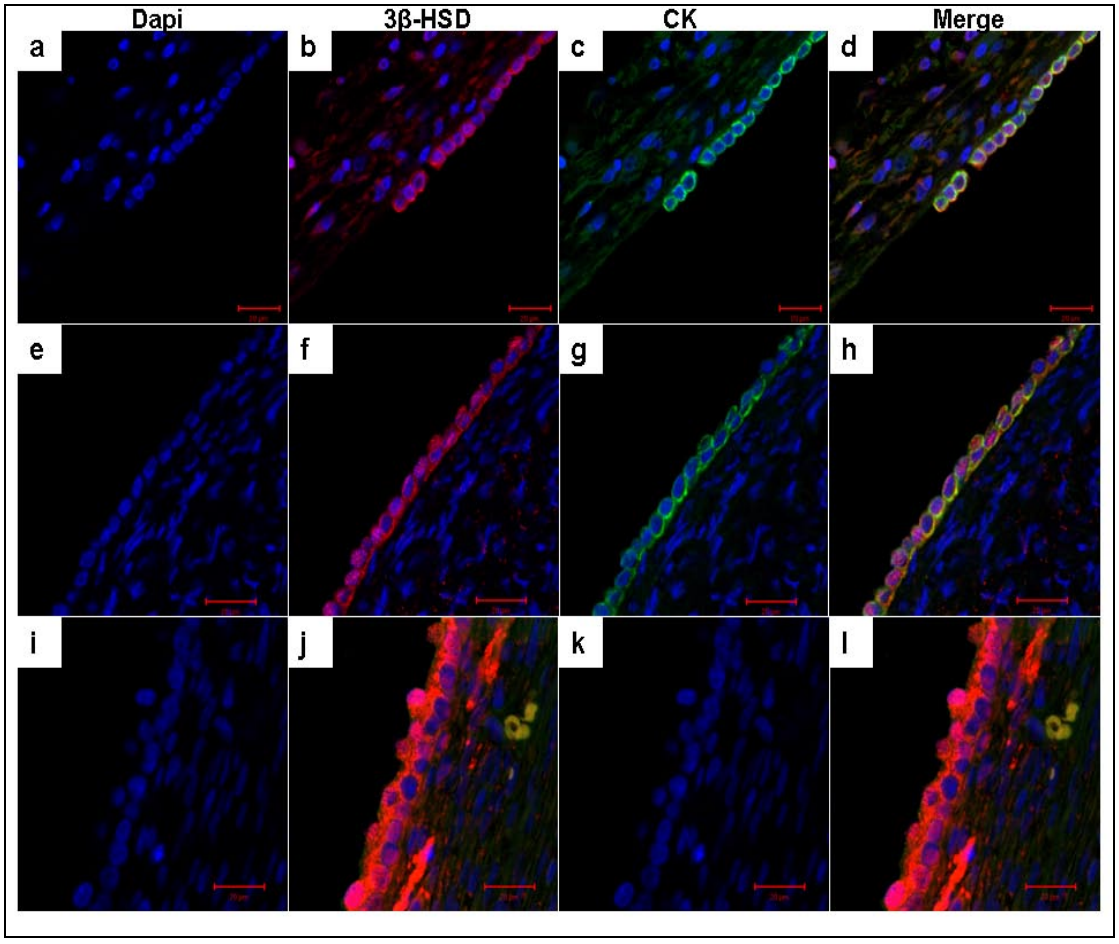


Figure 3.5: Double staining of 3β-HSD with epithelial-specific cytokeratins (CK). (a-d) Immunostaining with serial incubations with 3β-HSD (red) and cytokeratins 5, 6, 8, 17 (green) antibodies. Cytoplasmic staining of both antibodies was observed with no staining in stroma. (e-h) Double immunostaining with 3β-HSD (red) and cytokeratin 7 (green) showed epithelial-specific localisation of 3β-HSD. (i-l) Ovarian follicular cells were challenged with 3β-HSD (red) and cytokeratins (green). 3β-HSD was present to ovarian granulosa at high levels with no staining with cytokeratins. Nuclei were counterstained with Dapi (blue). Pictures were captured with Zeiss LSM 510 Meta Axiovert 100M confocal microscope at 40X magnification. Scale bars represent 20μm.

3.3.4 Expression of 3β-HSD in cultured hOSE cells

As shown in Section 3.3.3, 3β-HSD was expressed in the hOSE cellular compartment. However, as noted above, hOSE cell numbers at the time of collection were very low and so culture of such cells for at least two weeks was essential not only to get enough cells for further experimentation but also to balance the steroid milieu among samples that were collected at different stages of the menstrual cycle and might potentially reflect a variability among different patients. Therefore, prior investigation of the role and regulation of 3β-HSD in hOSE cells *in vitro*, an essential step was to establish that basal 3β-HSD functionality is maintained in our culture system. Regarding this, 3β-HSD protein and activity were measured in cultured hOSE cells using immunofluorescence and radiometric activity assays, respectively.

3.3.4.1 Expression of 3β-HSD protein in hOSE cell cultures

hOSE cells serum-depleted for 24h were subjected to serial incubations with 3β-HSD antibody and epithelial-specific cytokeratins (CK) (Fig. 3.6 a-d). The epithelial character of these cells (green) and their ability to express functional 3β-HSD (red) was revealed by overlapping staining of the two antibodies in cytoplasm. This suggests that our culture system gives epithelial cells of high purity that maintain expression of 3β-HSD. Dapi was used to stain nuclei. Representative pictures are shown.

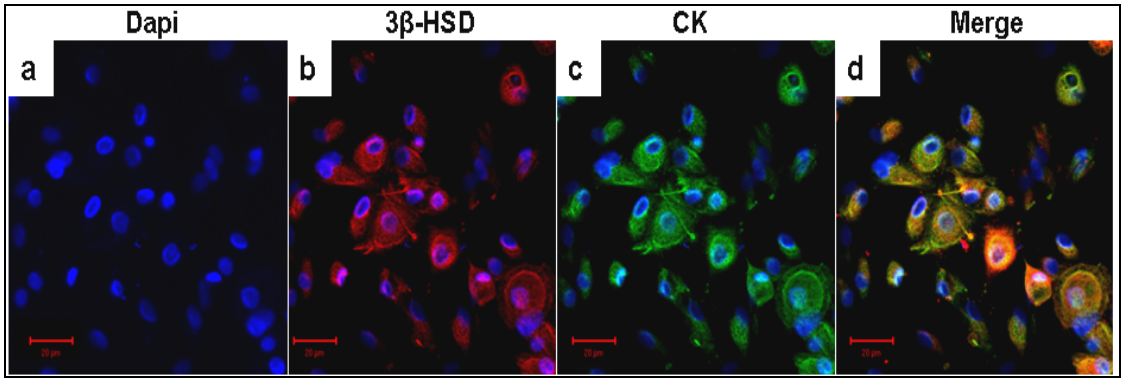


Figure 3.6: Double staining of 3β-HSD with epithelial-specific cytokeratins (CK) in cultured hOSE cells. (a-d) 3-week cultured hOSE cells were subjected to serial incubations with 3β-HSD (red) and cytokeratins 5, 6, 8, 17 (green) antibodies. Clear cytoplasmic staining of both antibodies shows epithelial character of those cells (c) and their capacity to express 3β-HSD. Nuclei were counterstained with Dapi (blue). Pictures captured with Zeiss LSM 510 Meta Axiovert 100M confocal microscope at 40X magnification. Scale bars represent 20μm.

3.3.4.2 Radiometric assay of 3β-HSD activity in hOSE cell cultures

Basal 3β-HSD activity in cultured hOSE cells was measured using a radiometric substrate conversion assay that involved measurement of [^{3}H]-progesterone after treatment of hOSE with [^{3}H]-pregnenolone as a substrate and separation according to their polarity/mobility on TLC (Fig. 3.7). Following a 3-week cell culture, 100,000 cells were re-plated and serum-depleted before investigating 3β-HSD activity *in vitro*. The hOSE cells of all patients tested displayed basal 3β-HSD activity. There was variability in activity levels among different patients (Fig. 3.7 A). However, these differences did not appear to correlate with the clinical profile of patients, although this was beyond the scope of this thesis. Similar data were also obtained with the use of DHEA as a substrate for 3β-HSD (data not shown). Representative radiographs of pregnenolone and progesterone mobility are illustrated (Fig. 3.7 B).

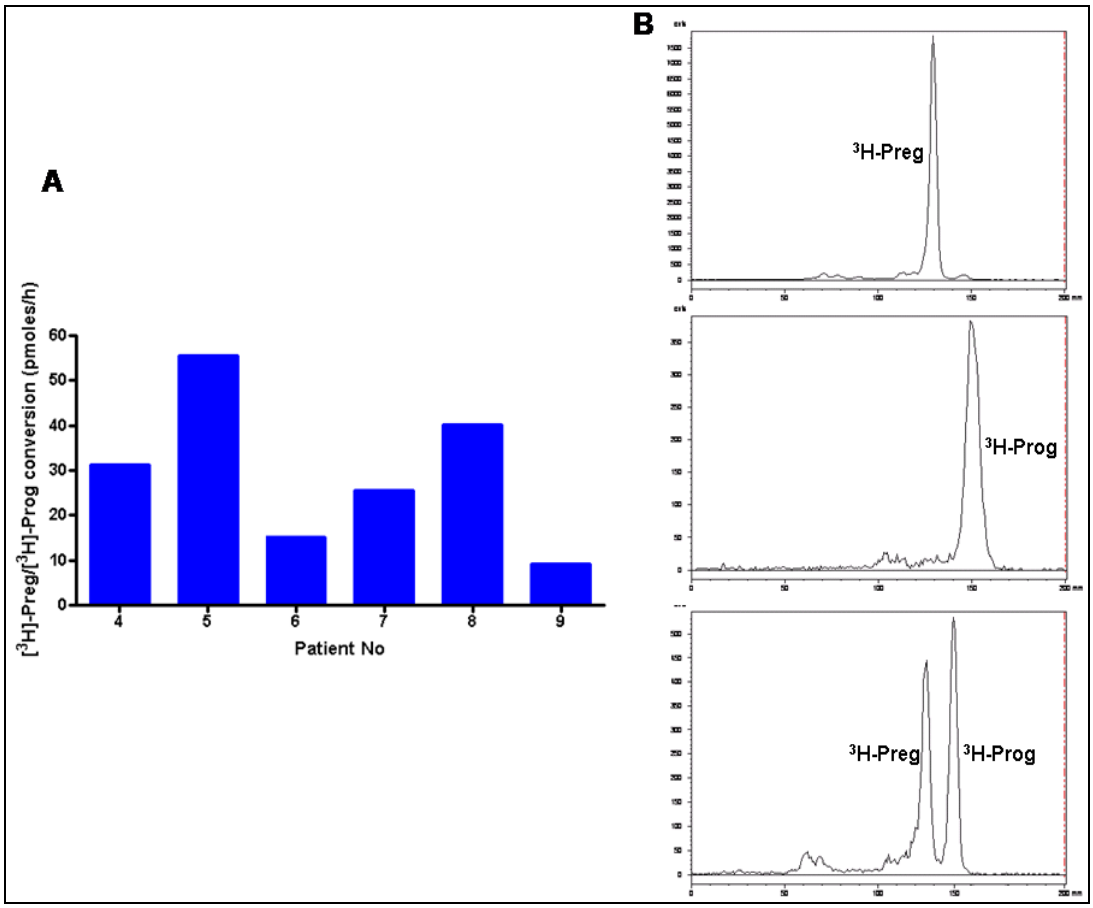


Figure 3.7: 3β-HSD activity in 6 distinct hOSE samples. A) After 3 weeks in culture, 100,000 hOSE cells/sample were re-plated and serum-depleted for 24h to measure basal 3β-HSD activity. All the samples were tested for their capacity to convert [^{3}H]-Preg to [^{3}H]-Prog, reflecting 3β-HSD activity B) Representative radiograph that shows [^{3}H]-Preg (upper radiograph) and [^{3}H]-Prog (centre radiograph) migration on silica-gel coated TLC plate. Lower radiograph illustrates the conversion of [^{3}H]-Preg to [^{3}H]-Prog in a representative hOSE sample.

3.3.5 3β-HSD isoform expression pattern in hOSE cell monolayers

The sections described above established expression and functionality of 3β-HSD in hOSE both *in vivo* and *in vitro*. However, the approaches described above were not conclusive of the isoform expression pattern of 3β-HSD isoforms. As noted in Chapter 1 (Secion 1.4.2.1), 3β-HSD1 and 3β-HSD2 share at least 93% similarity, making therefore the separation of the two isoforms in the same cellular compartment very difficult. Unfortunately, development of mouse monoclonal isoform-specific antibodies has not been successful to date. In order to investigate the gene regulation of 3β-HSDs in hOSE, it was essential to establish the isoform-expression pattern in this cell population. To achieve that, semi-quantitative and quantitative RT-PCR were performed with the use of isoform-specific primers as described in Chapter 2.

3.3.5.1 Semi-quantitative PCR (sqPCR)

DNase-treated RNA hOSE samples recruited from 5 patients were reversed transcribed and subjected to gene-specific PCR amplification to measure 3β-HSD1 and 3β-HSD2 transcript levels. Both gene products (Fig. 3.8, a, b) were expressed in 5 out of 5 hOSE samples. Samples transcribed without RT enzyme (RT –ve) were also run for each distinct sample to ascertain the absence of genomic contamination. The GAPDH gene was tested to control for the equal loading of initial cDNA template (c). 3β-HSD1 and 3β-HSD2 primer specificity was assessed with the inclusion of placental explants and ovarian lutein-granulosa cells, respectively. As expected, placenta was positive only for 3β-HSD1 mRNA (d), whereas lutein-granulosa expressed high levels of 3β-HSD2 mRNA (e). GAPDH mRNA amplification ascertained the equal loading and integrity of samples (f).

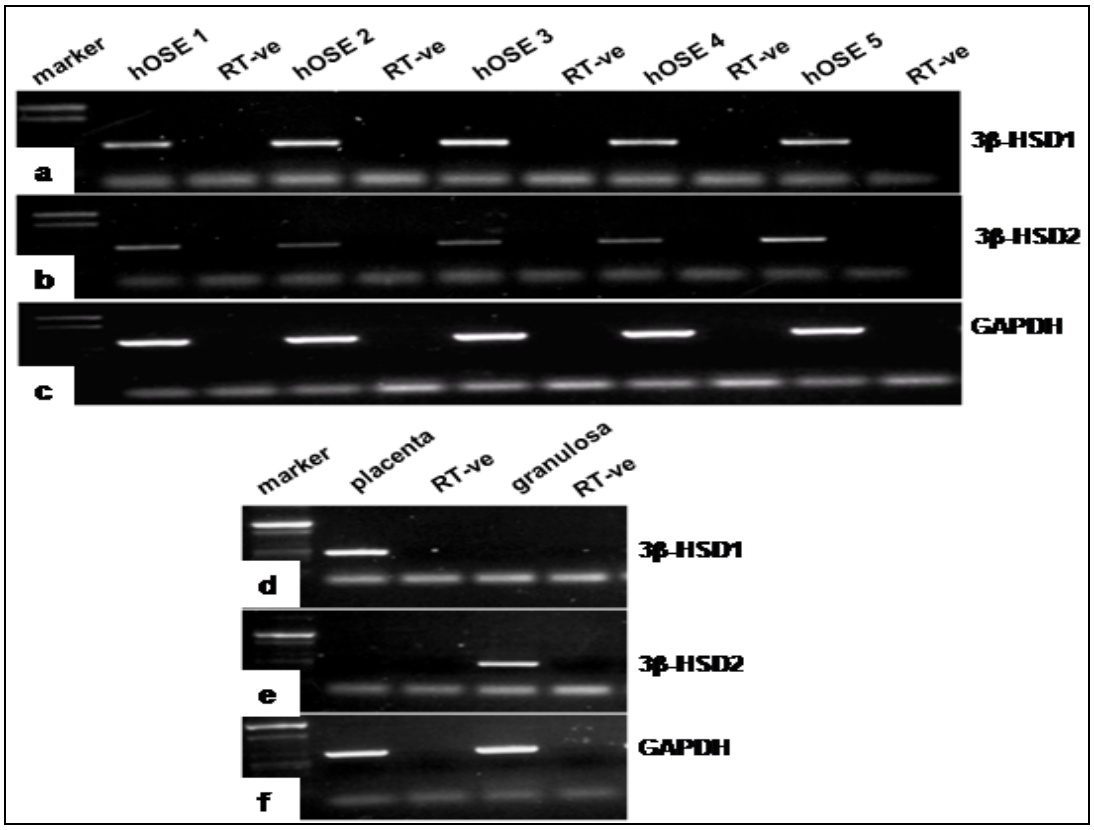


Figure 3.8: Semi-quantitative RT-PCR for 3β-HSD1 and 3β-HSD2 mRNA expression. hOSE samples from 5 patients were assessed for 3β-HSD1 and 3β-HSD2 mRNA expression (a, b). RT-ve was also run to control genomic contamination. Placenta (d) and ovarian lutein-granulosa cells (e) served as positive controls for 3β-HSD1 and 3β-HSD2 primers respectively. GAPDH mRNA was included to monitor cDNA quality/quantity (c, f).

3.3.5.2 Quantitative PCR (Taqman)

Because semi-quantitative PCR showed the presence of gene products for both 3β-HSD1 and 3β-HSD2 isoforms, absolute quantification of the products was performed with Taqman qPCR and the use of isoform-specific primers that had the same amplification efficiency. Moreover, in order to ascertain equal conditions of gene amplification, all samples were assessed relative to the house-keeping 18S gene, ensuring that the values obtained among different samples were tightly contained. Equal concentrations (200ng) of DNase-treated RNA of hOSE samples recruited from 7 patients were reversed transcribed and measured for 3β-HSD1 and 3β-HSD2 transcript levels. Both gene products (Fig. 3.9) were present in the hOSE of all patients tested (A, upper graph), although there was some variability of expression levels among patients (A, upper panel). 3β-HSD2 mRNA expression, however, appeared to be expressed at lower levels relative to 3β-HSD1 mRNA in all cases (A, upper panel). The combined data showed a mean 5-fold increase of 3β-HSD1 mRNA relative to 3β-HSD2 mRNA (A, lower panel; n=7, two-tailed paired student t-test, b=$p<0.001$). Noteworthy and albeit beyond the scope of the thesis, hOSE samples that were collected at the luteal phase of the menstrual cycle (Table 3.2, patients 14, 15, 17, 18) appeared to be correlated with higher 3β-HSD1 mRNA levels relative to samples collected at the follicular phase of the menstrual cycle (Table 3.2, patients 4, 16, 19); however this did not reach statistical significance (Fig. 3.9 B, upper panel). On the contrary, there was no such correlation regarding 3β-HSD2 mRNA levels (B, lower panel). 3β-HSD1 and 3β-HSD2 primer specificity was assessed with the inclusion of placental explants and ovarian lutein-granulosa cells respectively (C). Expectedly, placenta was positive only for 3β-HSD1 mRNA (C, upper panel), whereas lutein-granulosa expressed 70-fold higher levels of 3β-HSD2 mRNA relative to 3β-HSD1 mRNA (C, lower panel). Mean dCt values for hOSE cells, granulosa and placenta for 3β-HSD1 and 3β-HSD2 mRNA are illustrated in Table 3.3.

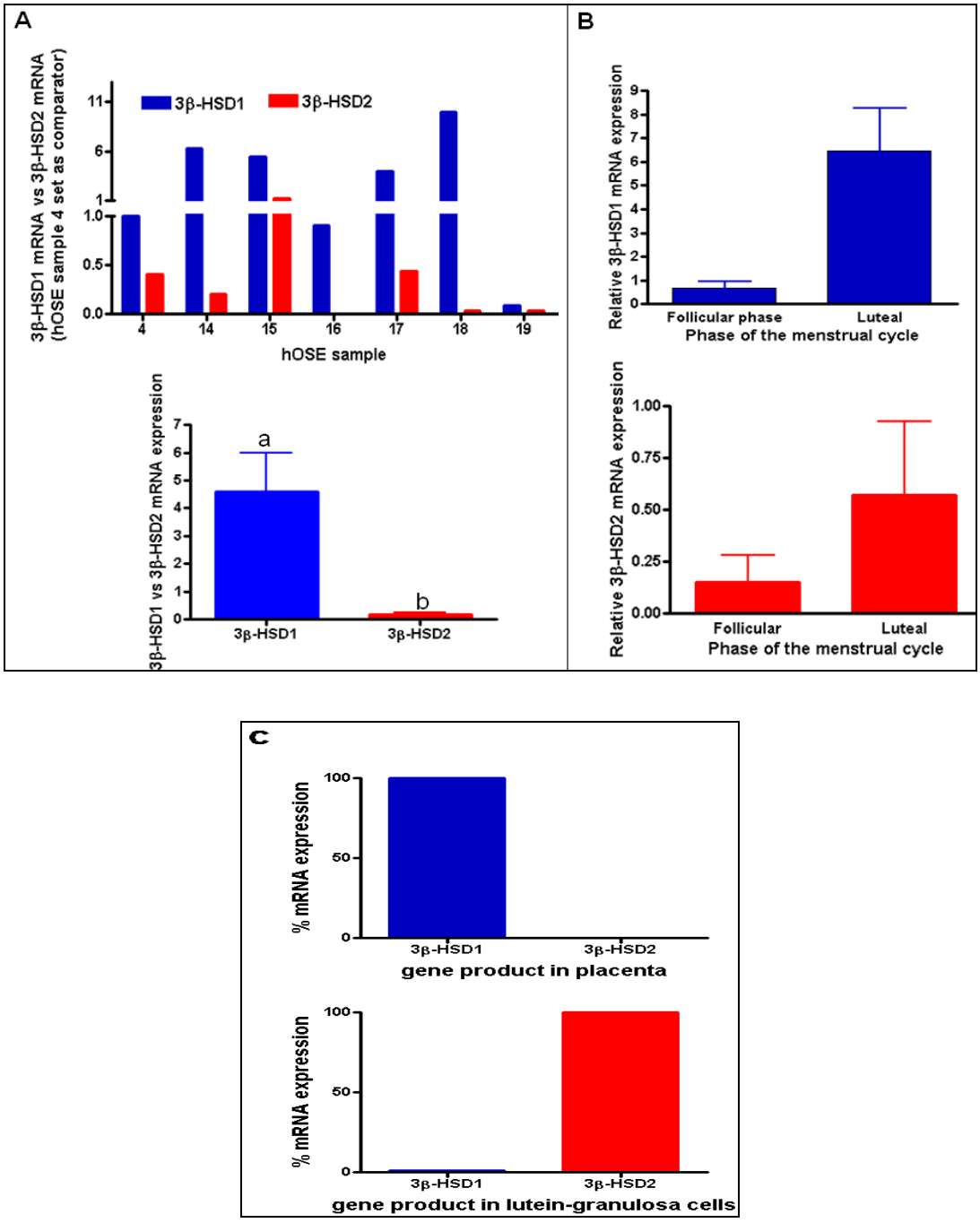


Figure 3.9: Quantitative RT-PCR for 3β-HSD1 and 3β-HSD2 mRNA expression. A) hOSE samples from 7 patients were assessed for 3β-HSD1 and 3β-HSD2 mRNA expression, using Taqman qPCR (n=7, b=p<0.001). B) Measurement of 3β-HSD1 or 3β-HSD2 mRNA levels and the phase of the menstrual cycle when samples were collected. C) Placenta and ovarian lutein-granulosa cells served as positive controls for 3β-HSD1 and 3β-HSD2 primers respectively.

Table 3.3 Mean dC_T values for Taqman Real-Time PCR

	3β-HSD1 mean dC_T	**3β-HSD2 mean dC_T**
Placenta	7.19	Not detected
Lutein-granulosa cells	14.32	7.89
hOSE cells	14.03	18.12

The dC_T values represent the PCR cycle that the target gene started to be accumulated relative to the internal reference 18S. Note that differences in dC_T values between different samples are presented at the logarithmic rather than the linear level

3.4 Discussion

Investigation of the pathophysiology of hOSE cells is important since this cellular compartment can be quite easily transformed and give rise to ovarian cancer (Auersperg *et al.* 2001, Runnebaum & Stickeler 2001). Although ovarian cancer is more common in post-menopausal women, it is believed to be a result of processes and genetic errors that are accumulated during reproductive age (Murdoch & McDonnel 2002) with 'incessant ovulation' the predisposing hypothesis of ovarian cancer (Fathalla 1971). Simultaneously, the ovarian environment at this age, develops mechanisms that protect the ovary from tumourigenesis. Elucidation of these mechanisms could contribute in the development of therapeutic strategies of ovarian cancer. Therefore, the study of normal pre-menopausal hOSE is an essential model to identify molecular mechanisms that could promote or protect from development of neoplasia.

Ovarian steroid environment is subject to periodic alterations during the menstrual cycle and it radically alters after menopause when most ovarian cancers appear, suggesting a possible role in the function of the OSE. Regarding this, it has been demonstrated that hOSE is a target of steroid metabolism and reception (Lau *et al.* 1999, Rembiszewska & Brynczak 1985). Intriguingly, progestogens and androgens, formation of both catalysed by 3β-HSD, exert apoptotic and cytoproliferative effects, respectively in human and sheep OSE (Bu *et al.* 1997, Edmondson *et al.* 2002, Murdoch *et al.* 2001) .

This chapter delineates some basic aspects for the optimal *in vitro* culture conditions of hOSE cells, a critical prerequisite for the validity of the data that are elaborated in the present and later chapters. Moreover, we provide direct evidence of notable expression of 3β-HSD mRNA, protein and activity in the hOSE layer of the human pre-menopausal ovary. We also show that our culture system can be successfully applied to study the *in vitro* role and regulation of 3β-HSD in this cell type.

Light phase-contrast microscopy was proved useful to study the *in vitro* growth and morphology of hOSE scrapings. Low numbers of cells recruited from each inoculum made impossible a comparison of growth potential between different hOSE samples. Nevertheless, it was noticed that the larger an inoculum was, the quicker it reached confluence. No correlation between cell growth potential and age of patient or stage of menstrual cycle when sample was collected was observed. Also, there were not any apparent differences in hOSE morphology and growth capacity among samples recruited from different harvesting methods. However, nothing of the above has been studied systematically by us or other groups and as such solid conclusions cannot be made.

As shown in the Results section of this chapter, confluent hOSE cells formed 'cobblestone'-like monolayers according to previous reports (Kruk *et al.* 1990). The fact that this cell morphology was maintained in subculture allowed significantly increased cell numbers and facilitated downstream analyses. It was consistently observed that primary cell inoculums that reached confluence within two weeks gave confluent cell monolayers following subculture for one more week, whilst hOSE scrapings with poor growth capacity senesced in subculture and could not be used further.

Epithelial-specific markers such as cytokeratins 5, 6, 8, 17 and 7 that were previously reported to stain hOSE cells *in situ* and *in vitro* but not stromal cells was a useful approach to ascertain epithelial purity of our cultures (Auersperg *et al.* 1994a, Czernobilsky *et al.* 1985, van Niekerk *et al.* 1991). Intriguingly, passaged cells also maintained cytokeratin staining, encouraging, therefore the use of those cultures for further experimentation (not shown). The latter was expected since subcultured cells were grown for a total of 4 weeks (2 weeks for primary inoculum plus 1 week in subculture plus 1 week maximum following re-plating for experimentation), consistent with the published period of time when those cells start to senesce or lose their epithelial character (Dyck *et al.* 1996). However, a solid assumption after that period of time cannot be made. Published data suggest maintenance of hOSE

monolayers and an average 70% keratin immunopositivity up to passage 3 with significant loss of cytokeratin staining after this period due to epithelio-mesenchymal transitions (Dyck *et al.* 1996).

Various experimental techniques showed the presence of functional 3β-HSD in hOSE cells obtained from pre-menopausal patients. A specific polyclonal rabbit antibody was used to immunolocalise 3β-HSD protein in hOSE layer of whole ovarian sections as well as in 3-week cultured hOSE cells. Interestingly, 3β-HSD immunolocalisation in hOSE was notable with the use of various immunohistochemical protocols, showing that the staining achieved was antibody-specific and not an artefact. Among various colourimetric protocols applied, antigen retrieval combined with microwave heating system appeared to give the most optimal protein staining, not only in hOSE but elsewhere in the ovary. Cytoplasmic staining of 3β-HSD was clearer however, when fluorescence immunohistochemistry was practised, suggesting that this method is probably more sensitive and accurate. Furthermore, the latter allowed the establishment of 3β-HSD co-localisation with epithelial-specific markers which further ascertained expression of this enzyme in hOSE *in vivo* and *in vitro*. Concomitant immunopositivity of pre-ovulatory follicles and corpus luteum for 3β-HSD further confirmed the data obtained. In support of this, activity assays of 6 distinct hOSE samples *in vitro* revealed capacity of those cells to convert [^{3}H]-pregnenolne to [^{3}H]-progesterone, reflecting active 3β-HSD in hOSE cells.

Whereas 3β-HSD expression has been reported in ovarian cancers (Abd-Elaziz *et al.* 2005), to our knowledge, these are the first data to show functional 3β-HSD protein and activity in the hOSE of the pre-menopausal ovary. 3β-HSD is at the top of the steroidogenic pathway and is considered a prerequisite for the generation of all active steroid hormones, namely mineralocorticoids, glucocorticoids, oestrogens, progestogens and androgens, all essential for body homeostasis. 3β-HSD is responsible for the generation of progesterone from pregnenolone as well as for androstenedione from DHEA. The latter can be subsequently reduced to more potent

androgens such as testosterone and 5α-DHT by 17β-HSD that has been shown to be present in hOSE (Rae *et al.* 2004b). Therefore, our data suggest that hOSE has all the dynamics required to actively participate in ovarian steroidogenesis and any deficiency at this point may result in ovarian disorder. Importantly, this becomes more complicated given the opposite effects of androgens and progestogens noted previously in hOSE (Bu *et al.* 1997, Edmondson *et al.* 2002, Murdoch *et al.* 2001). However, in physiology, maintenance of steroid balance is probably critical for the maintainence of homeostasis in this cellular compartment.

Another aspect of interest that arose from our immunohistochemical studies was the differences in immunostaining intensity among different cell types within the hOSE compartment. Columnar and rounded cells (A cells) appeared to be stained stronger for 3β-HSD than flattened cells (B cells) (Gillett *et al.* 1991), suggesting disparity in steroidogenic capacity of different cell types within the hOSE cell compartment and this might have biological significance in processes that precede pre-neoplastic transformation of those cells. Though, this aspect could not be investigated thoroughly since recruitment of ovaries with a completely intact hOSE cell layer was not practically possible. Intriguingly, it has been reported that the B cell zone was mainly encountered adjacent to ovulatory sites (stigma), suggesting that those cells might be a result of re-epithelisation following ovulation-associated healing of the cell surface and that they could particularly acquire neoplastic features (Gillett *et al.* 1991). It remains to be elucidated if loss of 3β-HSD is an acquired feature of pre-neoplastic transformation of OSE cells.

Since both antibody and activity studies are not 3β-HSD isoform-specific, we further analysed the pattern of 3β-HSD isoform expression at the mRNA level through semi-quantitative and quantitative gene-specific PCR. Semi-quantitative and quantitative RT-PCR showed that both 3β-HSD gene products are present to hOSE cells, with 3β-HSD1 mRNA predominating over 3β-HSD2 mRNA. The caveat here could be that we compared two genes assayed with specific but different sets of primers/probes. It should be noted, however, that both primers and probes used for

each separate isoform were purchased pre-validated, both recognised fragments that span between exons 2 and 3 and had the same amplification efficiency. Also, we confirmed specificity by using lutein-granulosa cells and placenta as positive controls for 3β-HSD2 and 3β-HSD1 mRNA, respectively. Expectedly, placenta did not express 3β-HSD2 at all, while lutein-granulosa cells expressed both isoforms, though 3β-HSD2 mRNA was at much higher levels (70-fold) (Rheaume *et al.* 1991). The isoform-expression pattern in hOSE, therefore, differs to what has been previously observed in the other cell compartments of the ovary, where 3β-HSD2 is the primary isoform expressed in female gonad. Preponderance of 3β-HSD1 mRNA to hOSE as opposed to the other ovarian compartments is notable since 3β-HSD1 has a higher catalytic efficiency than 3β-HSD2 (Rheaume *et al.* 1991). Given that the hOSE layer is considered to have limited capacity to produce steroids *de novo* from cholesterol, at least *in vitro* (Rae *et al.* 2004b), 3β-HSD1 predominance is consistent with expression of this isoform in peripheral non-steroidogenic tissues.

In summary, we have characterised further the cell culture system used for the study of hOSE cells. Also, the limitations of this cultural system along with the approaches we undertook to overcome low cell numbers without affecting validity of data have been presented. Moreover, we have shown that hOSE expresses notable levels of 3β-HSD protein both *in vivo* and *in vitro* that might well have a biological role in ovarian (patho)physiology. Intriguingly, studies at the transcriptional level suggested that the predominant isoform expressed is 3β-HSD1, contrasting with the principal expression of 3β-HSD2 that has been observed elsewhere in the female gonad. However, this is consistent with a preference of the expression of this isoform in peripheral non-steroidogenic tissues.

Chapter 4

Effects of cytokines on 3β-hydroxysteroid dehydrogenases in primary human ovarian surface epithelial cells

4.1 Introduction

4.1.1 Steroid-associated inflammatory action in the human ovarian surface epithelium

As stated in Chapter 1, the post-ovulatory wound healing cycles that the ovarian cell surface undergoes are regulated by a tightly controlled interplay among immune mediators such as cytokines and associated hormones including gonadotrophins and steroids. This multi-component machinery monitors the proliferation of genetically healthy hOSE cells, thereby securing the integrity of the ovarian cell surface following follicular rupture. Any cell that escapes from surveillance by this complex system can progress to neoplastic transformation and clonal expansion of a scarred cell, giving rise to EOC (Murdoch & McDonnel 2002). Identifying the mechanisms that physiologically control ovulation-associated wound healing could contribute to the development of new molecular markers for the diagnosis and/or treatment of EOC (Rae & Hillier 2005). In this regard, studies focusing on how hOSE cells recover after ovulation-associated inflammation have established a fundamental role of steroid pre-receptor metabolism and downstream steroid signalling in the scar-free post-ovulatory healing of hOSE. Glucocorticoid pre-receptor metabolism and subsequent glucocorticoid action through GR-α has been proved an essential pathway to alleviate hOSE cells from inflammatory cascades (Gubbay *et al.* 2004, Rae *et al.* 2004a, Rae *et al.* 2004b, Yong *et al.* 2002). Moreover, progesterone has been shown to suppress pro-inflammatory mediators such as COX-2 and also to induce poly(ADP)-ribose polymerase and polymerase-β, both involved in the inflammation-associated cytoproliferation of hOSE to repair the wound (Murdoch 1998, Murdoch *et al.* 2001, Rae *et al.* 2004a). As discussed in Chapters 1 and 3, 3β-HSD1 and 3β-HSD2 are the isoforms that regulate intracrine formation of progesterone in hOSE. Although the relevant contribution of the two isoforms to overall 3β-HSD activity cannot be predicted, total 3β-HSD activity monitors the bioavailability in hOSE of apoptotic and anti-inflammatory progesterone as well as of cytoproliferative androgens for signalling through PR and

AR, respectively (Edmondson *et al.* 2002, Rae *et al.* 2004a, Rae *et al.* 2004b). Regarding this, a screening of steroid-related markers showed that IL-1α suppressed 3β-HSD1 mRNA transcript levels suggestive that progesterone and androgen pre-receptor metabolism in hOSE was under inflammatory control (Rae *et al.* 2004b). However, no evidence has been reported concerning the expression and regulation of the 3β-HSD2 isoform in hOSE. Investigation of the regulation of both isoforms separately and as a whole is essential to elucidate further the nature of 3β-HSD in hOSE post-ovulatory wound healing processes.

As stated in Chapter 1, along with IL-1α that affects a series of pro-inflammatory and anti-inflammatory genes, including 3β-HSD1, in the ovulatory hOSE (Rae *et al.* 2004b), a number of other cytokines are also secreted pre- and post-ovulatory not only from hOSE itself but also from follicular and stromal cells. It is therefore quite reasonable to speculate that several cytokines could have paracrine or autocrine roles in 3β-HSD-associated regulation of hOSE prior to and after ovulation. For example, IL-6, TNF-α, IL-18 and GM-CSF are pro-inflammatory cytokines that are released pre-ovulatory and they might well affect local steroid biosynthesis during ovulation. Moreover, IL-4 and IL-10, two classic anti-inflammatory T lymphocyte-associated cytokines, are secreted peri- and post-ovulatory and might well mediate intracrine formation of steroids that mediate post-ovulatory responses. It is also of interest that all these cytokines or at least their cognate receptors have been identified in the ovarian tumour microenviroment, suggesting that they might exert tumourigenic or anti-tumourigenic responses in EOC and this may have profound implications in the aetiology and treatment of the disease.

4.1.2 Aim

Following establishment of functional expression of 3β-HSD1 and 3β-HSD2 in the human ovarian cell surface in Chapter 3, we shall investigate regulation of the two isoforms by the aforementioned cytokines that are naturally secreted pre-, peri- and/or post-ovulatory and could have a role in post-ovulatory tissue remodelling of hOSE.

4.2 Subjects and Methods

Investigation of the role of several inflammatory and anti-inflammatory cytokines was assessed using primary hOSE cell monolayers as described in Chapter 2. Precisely, 3.5 X 10^5 viable hOSE cells were plated in 6-well culture plates and were allowed to attach for 24h. Following serum-depletion for another 24h, cells were treated with serial doses of cytokines for 48h before cell harvest and homogenisation in cell lysis buffer. RNA purification, RNA quantity and quality analyses preceded reverse transcription and quantitative Real-Time PCR for 3β-HSD1 and 3β-HSD2 mRNA to assess relative levels between untreated control and cytokine-treated hOSE cells. Following establishment of the cytokine dose that had a maximal effect on transcriptional levels of the target genes after 48h in culture, primary hOSE cells were treated in a time-course manner (0h, 12h, 24h, 48h) with this cytokine. Subsequently, a fixed dose of cytokine treatment was used to measure total 3β-HSD protein and activity through western immunoblotting and 3β-HSD radiometric activity assay, respectively. The cytokines tested are illustrated in Table 4.1. The basic clinical profile of patients involved with the respective assays performed on each are presented in Table 4.2 and Table 4.3.

Table 4.1 Cytokine treatments on primary hOSE cell monolayers

Pro-inflammatory cytokines	**Doses tested (ng/mL)**	**Number of hOSE samples (n)**
IL-1α	0.02, 0.1, 0.5	n=4
TNF-α	0.1, 0.5, 1.0	n=4
IL-6	0.1, 0.5, 1.0	n=5
GM-CSF	0.1, 0.5, 1.0	n=3
IL-18	0.1, 0.5, 1.0	n=2
T lymphocyte-associated cytokines		
IL-4	0.02, 0.1, 0.5	n=4
IL-10	0.1, 0.5, 1.0	n=2

All cytokines were purchased from R&D systems (Abingdon Science Park, Abingdon, UK) lyophilised in PBS+0.2% BSA, aliquoted and stored at -80^{o}C. All treatments were inoculated in serum-free cell culture medium.

Table 4.2 Clinical profile of patients used for quantitative Real-Time PCR

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
21	7304	04/S1103/36	34	DiagLapar	Dysmenorrhoea	(7) Follicular	IL-1α
22	7318	04/S1103/36	51	TAHBSO	HMB	n/s	IL-1α
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Luteal	IL-1α
24	5505	04/S1103/36	48	TAH	HMB	(21) Luteal	IL-1α
21	7357	05/S1103/14	33	TAH	HMB	n/s	IL-1α (TR)
25	7304	04/S1103/36	34	DiagLapar	Dysmenorrhoea	(7) Follicular	IL-1α (TR)
26	7361	04/S1103/36	39	LapSter	Constant bleeding	n/s	IL-1α (TR)
27	9012	04/S1103/36	34	LapSter	Prophylactic	n/s	IL-1α (TR)
18	7246	04/S1103/36	46	TAHBSO	HMB	(16) Luteal	TNF-α
28	5289	04/S1103/36	23	DiagLapar	Dysmenorrhoea	(32) Luteal	TNF-α
19	7249	04/S1103/36	44	STAH	Dysmenorrhoea	(8) Follicular	TNF-α
29	7277	05/S1103/14	45	TAHBSO	Menorrhoea	n/s	TNF-α
18	7246	04/S1103/36	46	TAHBSO	Dysmenorrhoea	(16) Luteal	IL-6
30	7248	04/S1103/36	32	TAH	Dysmenorrhoea	(19) Luteal	IL-6
31	7296	1998/6/33	51	Oophere-ctomy	Prophylactic	n/s	IL-6
15	5410	05/S1103/14	36	LAVH	Dysmenorrhoea	(17) Luteal	IL-6
32	7308	05/S1103/14	31	TAH	HMB	(24) Luteal	IL-6
15	5410	05/S1103/14	36	LAVH	Dysmenorrhoea	(17) Luteal	IL-18
32	7308	05/S1103/14	31	TAH	HMB	(24) Luteal	IL-18
33	7247	04/S1103/36	46	TAH	Fibroids	(31) Luteal	IL-10
30	7248	04/S1103/36	32	TAH	Dysmenorrhoea	(19) Luteal	IL-10
4	5433	04/S1103/36	39	TAH	Cyclical pain	(5) Follicular	GM-CSF
16	5434	1998/6/33	41	TAHBSO	Fibroids	(3) Follicular	GM-CSF
34	5444	04/S1103/36	45	TAH	Fibroids	(18) Luteal	GM-CSF
20	7423	04/S1103/36	49	TAH	Fibroids	(30) Luteal	IL-4
35	7435	04/S1103/36	43	TAH	Dysmenorrhoea	(14) Luteal	IL-4
36	7437	04/S1103/36	32	DiagLapar	Pelvic pain	(1) Mens/Fol	IL-4
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	n/s	IL-4
6	5484	05/S1103/14	32	TAH	Prolapse	(24) Luteal	IL-4 (TR)
7	5497	04/S1103/36	39	TAH	Pelvic pain	(10) n/s	IL-4 (TR)
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	IL-4 (TR)
38	7314	04/S1103/36	43	Oophere-ctomy	HMB	n/s	IL-1α+IL-4
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	IL-1α+IL-4
39	7326	04/S1103/36	43	TAH	Fibroids	n/s	IL-1α+IL-4
12	7383	04/S1103/36	43	TAHBSO	Fibroids	n/s	IL-1α+IL-4
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	n/s	IL-1α+IL-4

See Table 4.3 for all abbreviations.

Table 4.3 Clinical profile of patients used for 3β-HSD protein and activity assays

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
7	5497	04/S1103/36	39	TAH	Dysmenorrhea	(10) n/s	IL-1α, IL-4 protein
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Luteal	IL-1α, IL-4 protein
12	7383	04/S1103/36	43	TAHBSO	Fibroids	(13) n/s	IL-1α, IL-4, IL-1+IL-4 protein
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	(9) n/s	IL-1α, IL-4, IL-1+IL-4 protein
4	5433	04/S1103/36	39	TAH	Cyclical pain	(5) Follicular	IL-1α, IL-4 TLC
9	7229	04/S1103/36	40	TAH	HMB	(2) Follicular	IL-1α, IL-4 TLC
5	5447	04/S1103/36	23	DiagLapar	HMB/pain	(4) n/s	IL-1α, IL-4 TLC
7	5497	04/S1103/36	39	TAH	Dysmenorrhoea	(10) n/s	IL-1α, IL-4 TLC
6	5484	05/S1103/14	32	TAH	Prolapse	(24) Luteal	IL-1α, IL-4 TLC
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Luteal	IL-1α, IL-4 TLC
38	7314	04/S1103/36	43	Oopherec-tomy	HMB	n/s	IL-1α, IL-4 TLC

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, STAH: sub-total abdominal hysterectomy, LAVH: laparoscopic assisted vaginal hysterectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, LapSter: laparoscopic sterilisation, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. TLC: thin layer chromatography, TR: time-response.

4.3 Results

4.3.1 Effects of pro-inflammatory cytokines on 3β-HSD1 and 3β-HSD2 mRNA in primary hOSE cells

4.3.1.1 Effect of IL-1α on 3β-HSD1 and 3β-HSD2 transcripts

Treatment of 4 individual hOSE cell monolayers with IL-1α *in vitro* in a dose-response manner for 48h resulted in suppression of 3β-HSD1 mRNA levels (Fig.4.1, upper panel). The suppression mode was dose-dependent with approximately 3-fold decrease when 0.1 and 0.5ng/mL of IL-1α was added (b=p<0.05, c=p<0.01). No effect was observed with the lowest concentration tested. On the other hand, IL-1α dose-dependently induced 3β-HSD2 mRNA with an at least 4-fold increase when 0.5ng/mL of IL-1α was added (Fig. 4.1, lower panel; b=p<0.05, c=p<0.01).

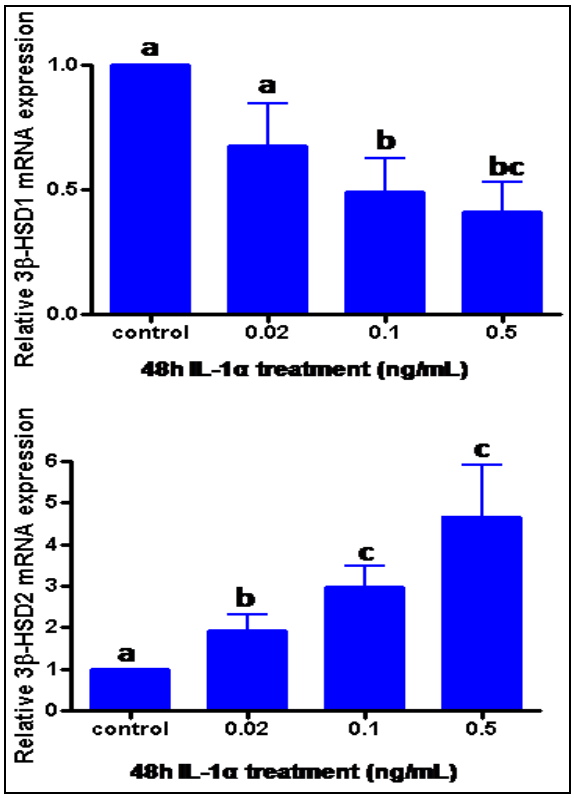


Figure 4.1: Effect of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 4 individual hOSE cell cultures. Primary hOSE cells were treated with increasing doses of IL-1α (0.02-0.5ng/mL) for 48h and expression of 3β-HSD1 mRNA (upper panel) and 3β-HSD2 mRNA (lower panel) were measured with Taqman qPCR (n=4, b=p<0.05, c=p<0.01)

4.3.1.2 Effect of TNF-α on 3β-HSD1 and 3β-HSD2 transcripts

Treatment of 4 individual hOSE cell monolayers *in vitro* with TNF-α in a dose-response mode for 48h did not affect expression levels of 3β-HSD1 or 3β-HSD2 mRNA (Fig.4.2, upper panel and lower panel, respectively). However, a trend towards down-regulation of 3β-HSD1 and up-regulation of 3β-HSD2 mRNA was noticed, although there was variability among separate hOSE samples and thus combined data did not reach statistical significance ($p>0.05$).

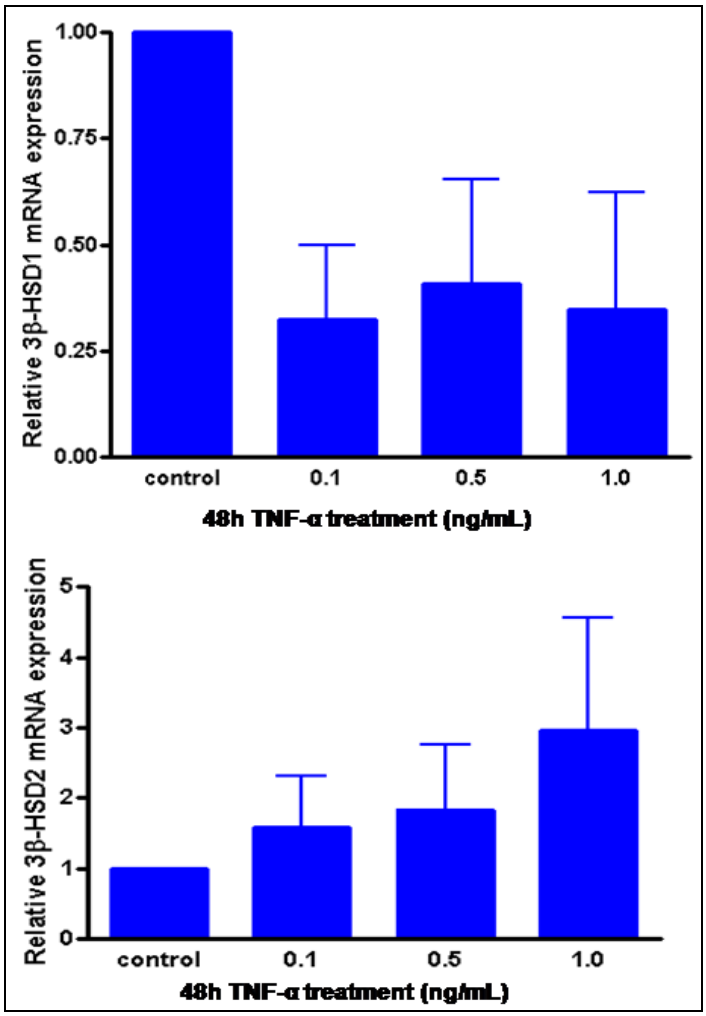


Figure 4.2: Effect of TNF-α on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 4 individual hOSE cell cultures. Cells were treated with increasing doses of TNF-α for 48h and effects on 3β-HSD1 and 3β-HSD2 mRNA were assessed (n=4).

4.3.1.3 Effect of IL-6 on 3β-HSD1 and 3β-HSD2 transcripts

Cell monolayers of hOSE cells collected from 5 separate patients were treated *in vitro* with serial doses of the inflammatory cytokine IL-6 for 48h. Following cell harvest and RNA/cDNA preparation, quantitative PCR showed that none of the 3β-HSD1 or 3β-HSD2 mRNA expression levels were affected relative to the untreated control samples (Fig. 4.3 upper and lower panels; $p>0.05$).

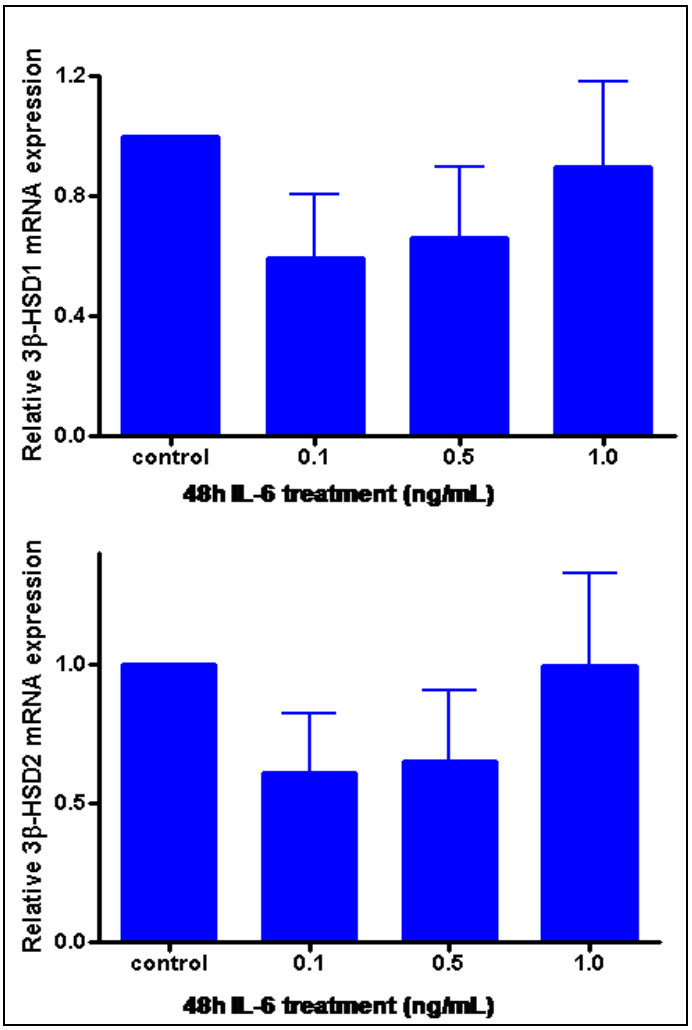


Figure 4.3: Effect of IL-6 on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 5 individual hOSE cell cultures. Treatment with serial doses of IL-6 for 48h was applied and then effects on 3β-HSD1 and 3β-HSD2 mRNA with Taqman qPCR were evaluated (n=5).

4.3.1.4 Effect of GM-CSF on 3β-HSD1 and 3β-HSD2 transcripts

Cell monolayers of hOSE cells collected from 3 separate patients were treated *in vitro* with serial doses of the inflammatory cytokine GM-CSF for 48h. Measurement of 3β-HSD1 and 3β-HSD2 mRNA expression levels in control untreated and treated samples did not show significant alterations in relative transcriptional expression levels of any of the target genes (Fig. 4.4 upper and lower panels; n=3, p>0.05), although a trend towards down-regulation of 3β-HSD2 mRNA was observed (Fig. 4.4 lower panel).

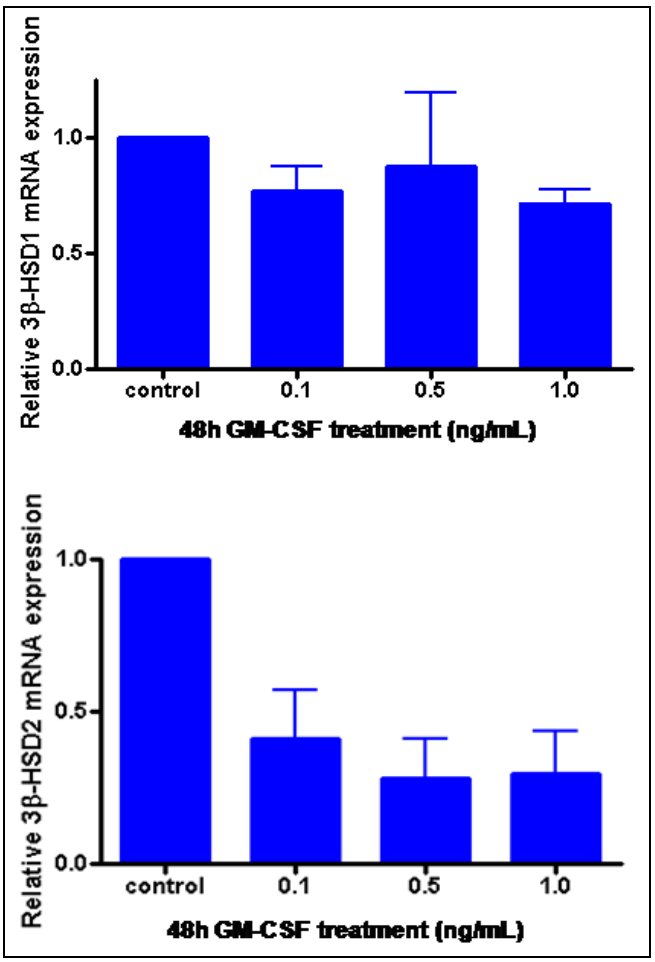


Figure 4.4: Effect of GM-CSF on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 3 individual hOSE cell cultures. Expression levels of 3β-HSD1 and 3β-HSD2 mRNA were measured after *in vitro* addition of serial doses of GM-CSF for 48h (n=3).

4.3.1.5 Effect of IL-18 on 3β-HSD1 and 3β-HSD2 transcripts

Cell monolayers of hOSE cells collected from 2 separate patients were treated *in vitro* with serial doses of the inflammatory cytokine IL-18 for 48h and relative 3β-HSD1 and 3β-HSD2 mRNA expression levels in control untreated and treated samples were measured with quantitative Real-Time PCR. Although insufficient replicates did not allow statistical analysis, IL-18 did not appear to affect basal expression levels of target genes at any dose tested (Fig. 4.5 upper and lower panels; n=2).

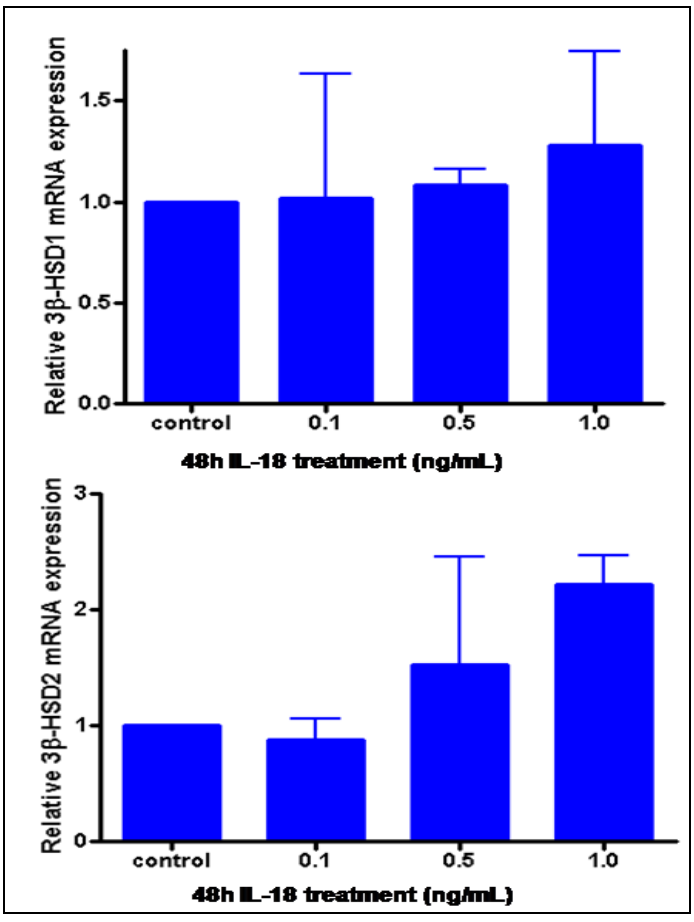


Figure 4.5: Effect of IL-18 on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 2 individual hOSE cell cultures. Measurement of 3β-HSD1 (upper panel) and 3β-HSD2 (lower panel) mRNA expression levels was performed after *in vitro* addition of serial doses of IL-18 for 48h. Insufficient replicates (n=2) did not allow statistical analysis.

4.3.2 Effects of T lymphocyte-associated cytokines on 3β-HSD1 and 3β-HSD2 mRNA in primary hOSE cells

4.3.2.1 Effect of IL-4 on 3β-HSD1 and 3β-HSD2 transcripts

We also tested the regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-4, a T lymphocyte-associated cytokine. Treatment of 4 distinct primary hOSE cell cultures with increasing doses of IL-4 (0.02-0.5ng/mL) for 48h resulted in substantial stimulation of both 3β-HSD1 and 3β-HSD2 mRNA expression levels (Fig. 4.6, upper and lower panels, respectively; n=4, b=$p<0.05$, c,d=$p<0.001$). This response was dose-dependent with an approximately 15-fold increase in 3β-HSD1 mRNA levels when 0.5ng/mL of IL-4 was added (Fig. 4.6, upper panel). Regarding 3β-HSD2 mRNA expression levels, IL-4 dose-dependently led to a 65-fold enhancement when 0.5ng/mL of IL-4 was added (Fig. 4.6, lower panel).

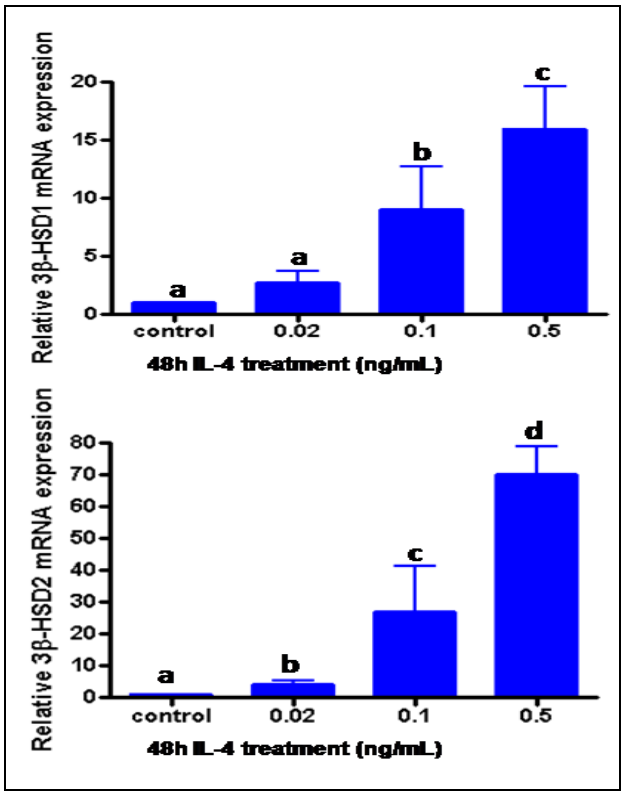


Figure 4.6: Effect of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 4 individual hOSE cell cultures. IL-4 treatment in a dose-dependent manner was applied for 48h and effects on 3β-HSD1 mRNA (upper panel) and 3β-HSD2 mRNA (lower panel) expression levels were tested with Taqman Real-Time PCR (n=4, b=$p<0.05$, c,d=$p<0.001$).

4.3.2.2 Effect of IL-10 on 3β-HSD1 and 3β-HSD2 transcripts

A separate T lymphocyte-associated cytokine that we used to perturb 3β-HSD1 and 3β-HSD2 transcriptional regulation was IL-10. Treatment of 2 distinct hOSE cell cultures with this cytokine for 48h did not show any changes on either 3β-HSD1 or 3β-HSD2 mRNA expression levels (Fig. 4.7, upper and lower panels, respectively). Lack of sufficient replicates did not permit a statistical analysis of these data.

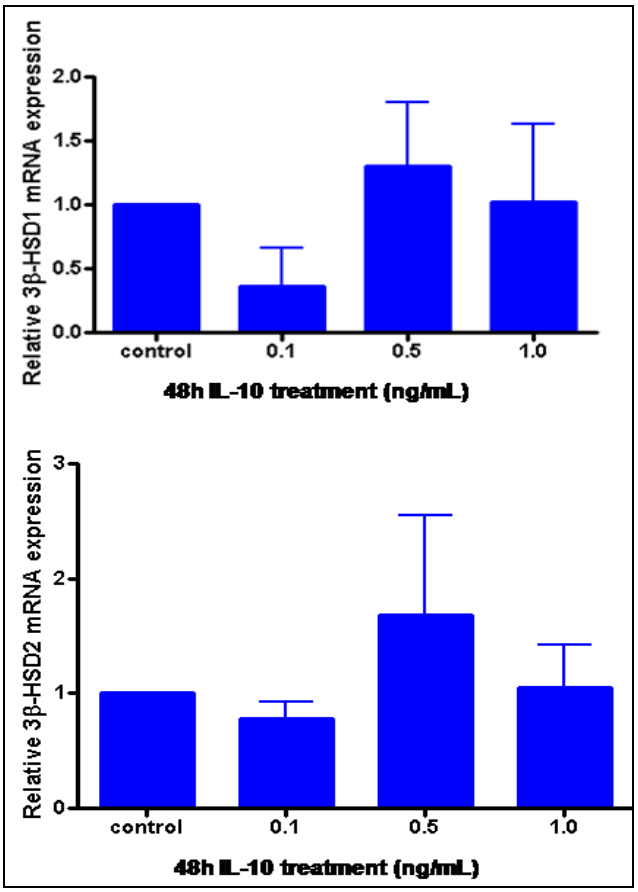


Figure 4.7: Effect of IL-10 on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 2 individual hOSE cell cultures treated with sequential doses of IL-10 for 48h. Effects on 3β-HSD1 (upper panel) and 3β-HSD2 (lower panel) mRNA levels were assessed with Taqman qPCR (n=2).

4.3.3 Effects of increasing incubation time of IL-1α and IL-4 on 3β-HSD1 and 3β-HSD2 transcripts in primary hOSE cells

After establishment of the cytokines that might regulate 3β-HSD1 and 3β-HSD2 transcript levels, cytokine effects on 3β-HSD transcriptional regulation in time-course studies were investigated further.

4.3.3.1 Time-dependent studies of IL-1α on 3β-HSD1 and 3β-HSD2 transcripts

As shown above, treatment of hOSE cells with serial doses of IL-1α for 48h, differentially affected 3β-HSD1 and 3β-HSD2 mRNA. The same effect was observed when a fixed dose of IL-1α (0.5ng/mL) was added in hOSE cell monolayers in a time-response mode. Intriguingly, IL-1α-suppression of 3β-HSD1 mRNA was achieved progressively with a significant 8-fold inhibition after addition of IL-1α for 48h (Fig. 4.8, upper panel; n=4, b=$p<0.05$). On the other hand, an IL-1α stimulatory effect on 3β-HSD2 mRNA was more rapid with a significant up-regulation at the 12h time-point with no further induction within time (Fig. 4.8 lower panel; n=4, c=$p<0.01$).

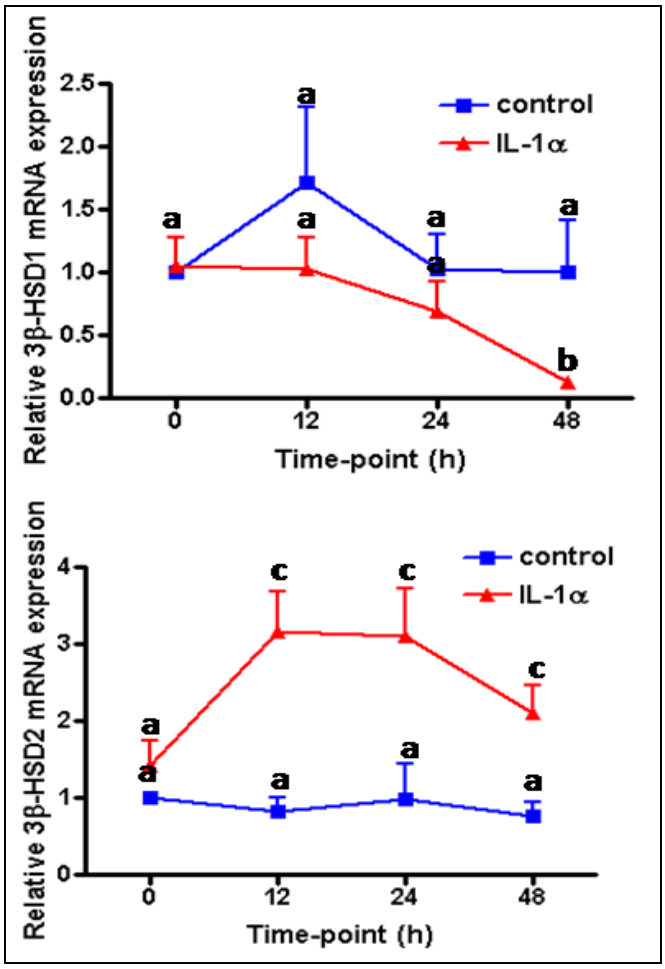


Figure 4.8: Time-dependent studies of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 4 separate experiments. IL-1α treatment (0.5ng/mL) was applied in a time-dependent mode (12, 24, 48h). Effects on 3β-HSD1 mRNA (upper panel) and 3β-HSD2 (lower panel) mRNA were measured with Taqman qPCR (n=4, b=$p<0.05$, c=$p<0.01$).

4.3.3.2 Time-dependent studies of IL-4 on 3β-HSD1 and 3β-HSD2 transcripts

Time-course (12, 24, 48h) studies with 0.5ng/mL IL-4 further supported its substantial stimulatory effect on both 3β-HSD transcripts (Fig. 4.9). Intriguingly, the effect was achieved by the 12h time-point with an apparent steady state achieved through to the 48h time-point (Fig. 4.9, upper and lower panels, respectively; n=3, b=p<0.01, c=p<0.001). IL-4 treatment of hOSE cells led to a mean 15-fold increase of 3β-HSD1 mRNA (Fig. 4.9, upper panel) and a mean 55-fold up-regulation of 3β-HSD2 mRNA levels.

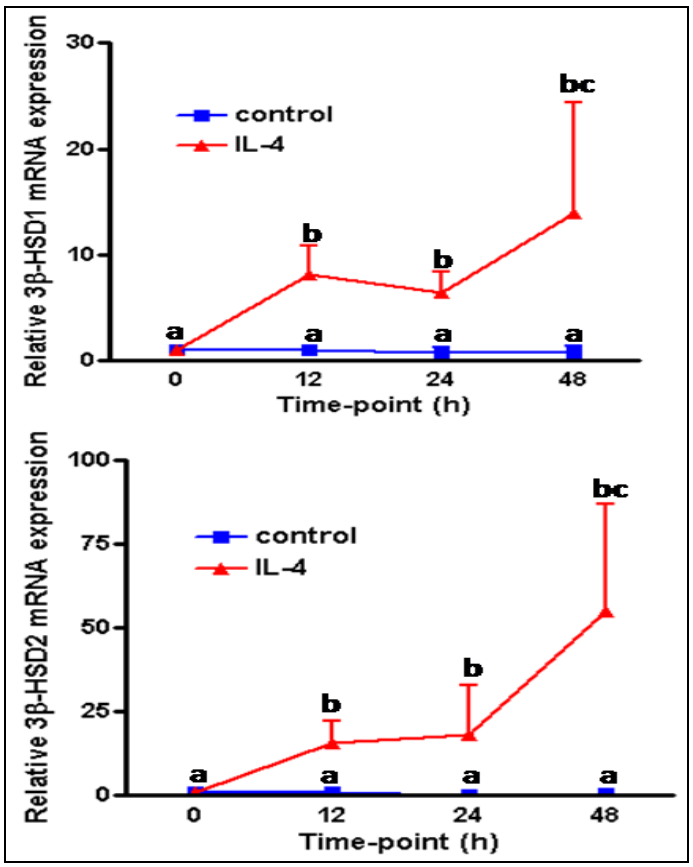


Figure 4.9: Time-dependent studies of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA. Combined data of 3 independent experiments. Treatments with IL-4 (0.5ng/mL) for 12, 24 and 48h took place and 3β-HSD1 (upper panel) and 3β-HSD2 (lower panel) mRNA levels were measured with Taqman qPCR (n=4, b=p<0.01, c=p<0.001).

4.3.4 Effects of IL-1α and IL-4 on 3β-HSD protein and activity in primary hOSE cells

Following investigation of individual responses of IL-1α and IL-4 to 3β-HSD1 and 3β-HSD2 mRNA, our next step was to establish the differential cytokine effects on total 3β-HSD protein and activity, since intracrine bioavailability of steroids in hOSE is controlled by total 3β-HSD action. As such, treatment of hOSE collected from 7 patients with 0.5ng/mL IL-1α for 48h did not significantly affect 3β-HSD protein (Fig. 4.10, upper panel) or total 3β-HSD activity (Fig. 4.10 lower panel). On the other hand, 0.5ng/mL IL-4 treatment for 48h significantly induced 3β-HSD protein (Fig. 4.10, upper panel) as well as progesterone formation by pregnenolone in hOSE, thereby reflecting an increase in total 3β-HSD activity (Fig. 4.10, lower panel). Similar data were also obtained with treatment with DHEA as a substrate for 3β-HSD activity (data not shown). Human placenta and human adrenocortical H295 cells (Fig. 4.10, upper panel) were used as positive controls for 3β-HSD1 and 3β-HSD2 proteins, respectively.

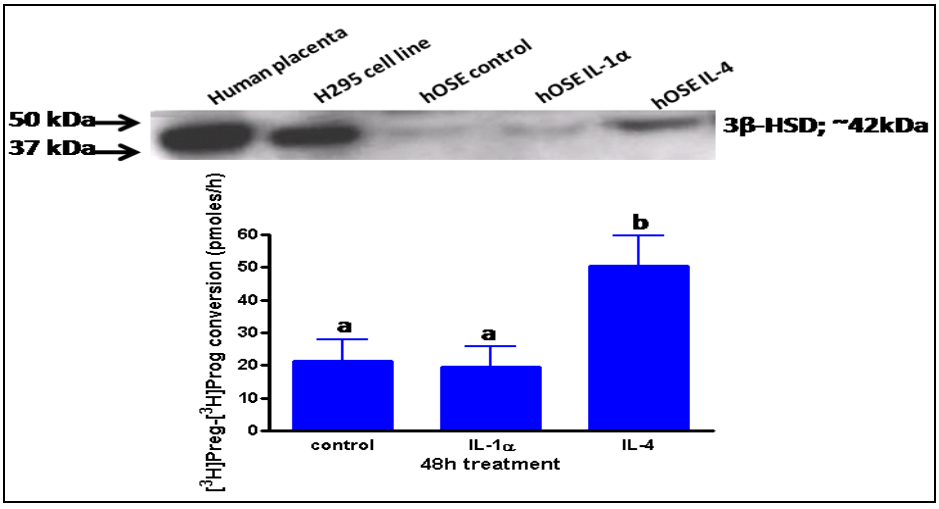


Figure 4.10: IL-1α and IL-4 effects on total 3β-HSD protein and activity in hOSE cells. Western immunoblotting was performed to measure 3β-HSD protein after treatment of hOSE cells with IL-1α and IL-4 (upper panel). 3β-HSD activity assay was performed after treatment of 7 independent hOSE cell cultures with IL-1α and IL-4 (lower panel; n=7, b=$p<0.001$).

4.3.5 Effects of IL-4 plus IL-1α co-treatment on 3β-HSD1 and 3β-HSD2 transcripts and 3β-HSD protein in primary hOSE cells

4.3.5.1 Morphology of hOSE cell monolayers after treatment with IL-4 in the presence or absence of IL-1α

Following the establishment of cytokine effects on 3β-HSD mRNA and protein, we then asked if there was any synergy between IL-1α and IL-4 signalling. To fulfil this, we co-treated hOSE cell monolayers with IL-1α plus IL-4 (0.5ng/mL) for 48h. Bright-field microscopy allowed us to monitor morphological changes of hOSE cells before and after treatments (Fig. 4.11). Relative to the untreated control cells, challenge of the cells with IL-1α for 48h affected hOSE cell morphology. Precisely, cells looked more elongated, displaying a fibroblast-like appearance, typical of regenerative hOSE cells. IL-4 treatment for 48h did not affect the hOSE cell epithelial phenotype *in vitro*. In IL-1α plus IL-4 co-treated cell monolayers, there was a mixture of epithelial and fibroblast-like cells.

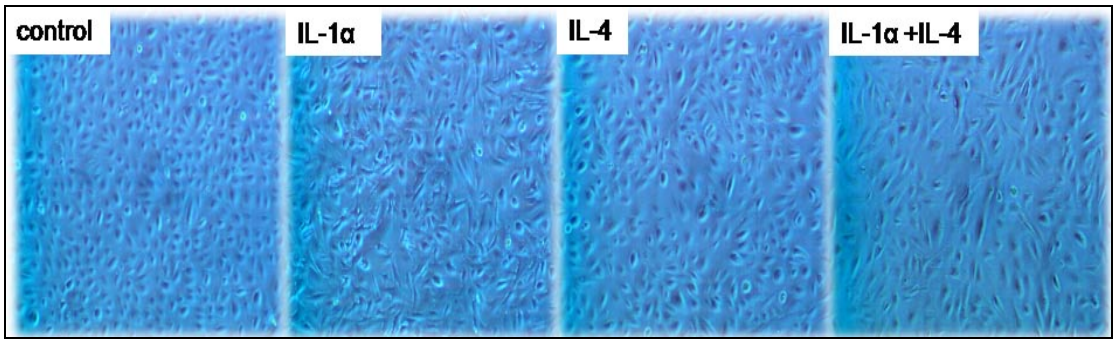
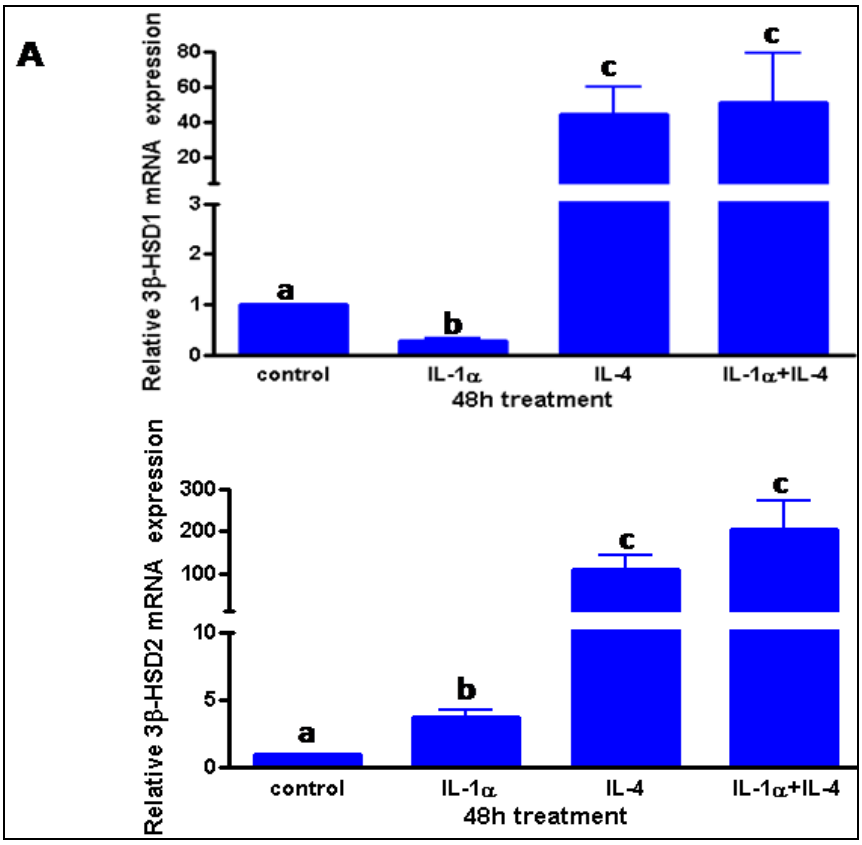


Figure 4.11: Morphology of hOSE cells treated with IL-1α, IL-4 or both. Phase contrast microscopy of live cell monolayers in the presence or absence of IL-1α, IL-4 or both cytokines (0.5ng/mL) for 48h. Morphology of cells in untreated hOSE cells (1st capture, control), treated with IL-1α (2nd capture), IL-4 (3rd capture) or IL-1α plus IL-4 are depicted (4th capture).

4.3.5.2 Effects of IL-4 co-treated with IL-1α on 3β-HSD1 and 3β-HSD2 transcripts along with total 3β-HSD protein

To investigate further a potential synergy between pro-inflammatory IL-1α and anti-inflammatory IL-4 in 3β-HSD1 and 3β-HSD2 transcriptional regulation (Fig. 4.12 A) along with total 3β-HSD protein (Fig. 4.12 B), we measured relative mRNA and protein levels of target genes in treated and untreated hOSE cells. At the transcriptional level, IL-4 (0.5ng/mL) appeared to override the IL-1α response (0.5ng/mL), as there was not any additive effect in co-treated cells (Fig. 4.12 A upper and lower panels; n=5, b=$p<0.05$, c=$p<0.001$). However, co-treatment of hOSE cells with IL-1α and IL-4 for 48h significantly increased total 3β-HSD protein compared to IL-4-induced total 3β-HSD (Fig. 4.12 B).



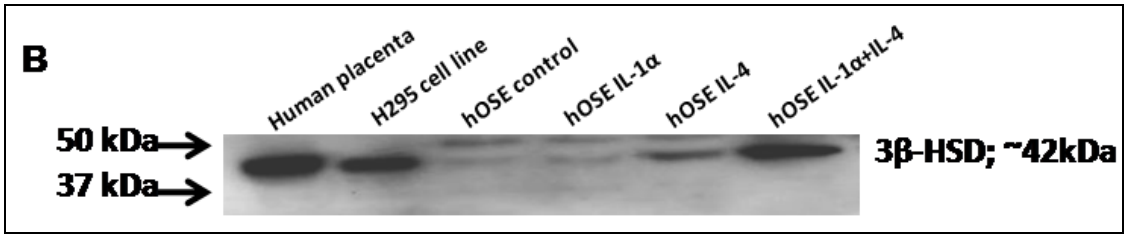


Figure 4.12: Effects of IL-1α and IL-4 co-treatment on 3β-HSD mRNA and protein. A) Combined data of 5 independent experiments. Treatment of hOSE cells with IL-1α plus IL-4 (0.5ng/mL) for 48h were performed. Effects on 3β-HSD1 and 3β-HSD2 mRNA levels were measured by Taqman qPCR (n=5, b=$p<0.05$, c=$p<0.001$). B) Representative western immunoblotting of co-treated hOSE cells with IL-1α and IL-4 (0.5ng/mL) for 48h to measure total 3β-HSD protein.

4.3.6 Summary of Results

4.3.6.1 Summary of data obtained through Taqman qPCR

Table 4.4 Summary of cytokine effects on 3β-HSD1 and 3β-HSD2 mRNA

Pro-inflammatory cytokines	**3β-HSD1 mRNA**	**3β-HSD2 mRNA**
IL-1α	↓	↑
TNF-α	n/s	n/s
IL-6	n/s	n/s
GM-CSF	n/s	n/s
IL-18	n/s	n/s
T lymphocyte-associated cytokines		
IL-4	↑	↑
IL-10	n/s	n/s
Co-treatment		
IL-1α+IL-4	↑	↑

n/s: non-significant; ↑: up-regulation; ↓: down-regulation

4.3.6.2 Summary of data obtained at the protein and activity level

Table 4.5 Summary of cytokine effects on total 3β-HSD protein and activity

Cytokines	**3β-HSD protein**	**3β-HSD activity**
IL-1α	**n/s**	**n/s**
IL-4	↑	↑
IL-1α+IL-4	↑	-

n/s: non-significant; ↑: up-regulation; -: not tested

4.4 Discussion

In Chapter 3, we established that both 3β-HSD isoforms are present in hOSE cells but we did not distinguish their relative functional importance. In the present Chapter, we elaborate the regulation of both 3β-HSD isoforms by inflammatory-associated mediators, reflecting therefore the intracrine formation of androgens and progesterone in hOSE under the control of these immune factors. We screened the effects of a panel of 'inflammatory' and 'anti-inflammatory' cytokines on 3β-HSD1 mRNA, 3β-HSD2 mRNA and total 3β-HSD protein and activity. Intriguingly, all the cytokines tested are naturally secreted in the ovary pre- and post-ovulatory and are also part of the ovarian tumour microenvironment. Moreover, mRNAs for all the pro-inflammatory cytokines assessed, namely IL-1α, TNF-α, IL-6, IL-18 and GM-CSF are expressed in hOSE, primary EOC and cell lines along with their cognate receptors (Burke *et al.* 1996, Lidor *et al.* 1993, Merogi *et al.* 1997, Wang *et al.* 2002b, Ziltener *et al.* 1993), suggesting autocrine and paracrine loops thereof in hOSE and ovarian cancer cells. On the other hand, the T lymphocyte-associated cytokines assessed, namely IL-4 and IL-10 do not appear to be secreted by hOSE or malignant ovarian cells, although cognate receptors are present (Burke *et al.* 1996). This implies possible paracrine roles of the latter in the normal and malignant epithelial ovarian cells.

We identified two cytokines, the pro-inflammatory IL-1α and the T lymphocyte- associated cytokine IL-4, to differentially mediate 3β-HSD action, at least *in vitro*, in pre-menopausal hOSE cells. Previously, a microarray analysis focusing on candidate inflammatory-associated genes showed that IL-1α suppressed 3β-HSD1 mRNA (Rae *et al.* 2004b). Herein, we confirmed this observation but we also demonstrated for the first time that IL-1α induced 3β-HSD2 mRNA. A novel finding was that the differential effects of IL-1α in the two 3β-HSD mRNA isoforms resulted in maintenance of basal levels of total 3β-HSD protein and activity, at least in the treatment window applied. Although 3β-HSD1 mRNA appeared to predominate in quantity over 3β-HSD2 mRNA levels, the latter appeared to be

equally functional and rapidly induced under particular circumstances *i.e.* inflammation and ovulation. This in turn could restore progressive but significant steroid biosynthesis attenuation caused by IL-1α-suppressed 3β-HSD1 mRNA, enabling therefore hOSE cells to secure bioavailability of steroid synthesis during inflammatory ovulation and post-ovulatory tissue remodelling. This might well represent an efficient mechanism of hOSE cells to protect cell integrity during post-ovulatory repair and could also explain in part why ovulation is a scarless inflammatory event. Precisely, a decrease of 3β-HSD1 mRNA by IL-1α is suggestive that the initiation of ovulation requires minimal local steroid biosynthesis. Concomitantly, IL-1α-induced 3β-HSD2, favouring synthesis of active ligands for cognate nuclear steroid receptors, could be a functional post-ovulatory mechanism to counteract ovulation-associated inflammation and minimise susceptibility of tissue to genetic damage. Androgens, such as mibolerone, a synthetic androgen that specifically couples to AR, have been shown to induce cell proliferation and inhibit cell death (Edmondson *et al.* 2002), whereas progesterone has been shown to be anti-inflammatory (Rae *et al.* 2004a), apoptotic (Bu *et al.* 1997, Murdoch & Van Kirk 2002) and to inhibit cell growth (Ivarsson *et al.* 2001a) in hOSE and ovarian cancer. Therefore, androgen synthesis may be essential for post-ovulatory repair that involves DNA cell proliferation, whereas associated progesterone synthesis might be vital to nullify the proliferation of genetically damaged cells.

A caveat on the studies regarding treatment of hOSE cells with IL-1α is that in the total 3β-HSD protein and activity assays, we examined responses over a fixed incubation time of the cytokine. It would be intriguing to investigate if total 3β-HSD protein and activity levels were influenced by IL-1α in response to time in culture. Based on transcriptional studies of IL-1α in response to time, we would expect an induction of total 3β-HSD activity at early time-points, as a result of the 3β-HSD2 isoform, followed by progressive restoration to the basal levels due to attenuated 3β-HSD1 mRNA at a later time. We were not able to perform these studies due to limited primary cell availability. However, this weakness could be overcome by the fact that we used primary hOSE cell samples from distinct patients and not a single

cell line to prove reproducibility of responses, thereby taking into account potential variability among patients. As such, this could better reflect physiological responses of hOSE *in vivo*.

The 'anti-inflammatory' cytokine IL-4 strongly stimulated 3β-HSD1 mRNA and 3β-HSD2 mRNA as well as total 3β-HSD protein and activity in normal hOSE cells. Up-regulation of 3β-HSD mRNA and activity, enhancing progesterone and androgen biosynthesis, might be part of *in vivo* post-ovulatory healing and repair mechanisms. At this time, controlled proliferation is essential for integral regeneration of the ovarian cell surface. As referred to earlier, androgens are cytoproliferative for hOSE, thereby important in post-ovulatory healing (Edmondson *et al.* 2002). However, as discussed in Chapter 1, an androgenic dominant environment could promote EOC due to uncontrolled cell growth and thus clonal expansion of transformed OSE cells (Murdoch & McDonnel 2002, Risch 1998). To prevent this, hOSE physiologically has developed mechanisms to monitor the death of damaged cells. Mechanisms that promote formation and downstream signalling of apoptotic and anti-proliferative progesterone could fulfil this fundamental role (Bu *et al.* 1997, Murdoch 1998, Murdoch & Van Kirk 2002, Rae *et al.* 2004a). Therefore, IL-4-induced 3β-HSD implies an anti-proliferative mechanism in hOSE. Intriguingly, hOSE cells have no capacity to secrete IL-4 *de novo* but they do express high levels of IL-4 receptor (Burke *et al.* 1996, Ziltener *et al.* 1993). The main source of IL-4 secretion in the pre-menopausal ovary is the peripheral blood mononuclear cells that infiltrate the peri-ovulatory follicle and later the corpus luteum. IL-4 is highly up-regulated at the luteal phase of the menstrual cycle when progesterone is also at its peak (Hashii *et al.* 1998). Accordingly, during the progestogenic luteal phase, when OSE repair takes place, genetically scarred cells become committed to die (Murdoch & Van Kirk 2002b). This paracrine mechanism might well be partially mediated by the local induction of 3β-HSD in hOSE due to elevated IL-4 secretion in ovarian environment. It is also interesting that IL-4 levels are increased in the corpus luteum of pregnancy (Hashii *et al.* 1998) which is consistent with the protective role that pregnancy and multiparity have against ovarian cancer (Adami *et al.* 1994). In

support of this and similarly to pregnancy, it has been shown that progesterone in the presence or absence of IL-4 treatment inhibits Th-1 development of T cells and promotes their differentiation into Th-2 cells that have a fundamental role in alleviation of an inflammatory environment (Miyaura & Iwata 2002).

Given the differential effects of the pro-inflammatory IL-1α and anti-inflammatory IL-4 in hOSE, we next investigated if there is any cross-talk between them in the regulation of 3β-HSD1 mRNA, 3β-HSD2 mRNA and total protein. We achieved that by setting up co-treatment studies in primary hOSE cell cultures. Morphological observations suggested that IL-1α impacts upon hOSE phenotype, as propagation of cells in its presence displayed a fibroblast-like appearance typical of epithelio-mesenchymal transitions the hOSE undergoes during ovulation-associated regeneration (Auersperg *et al.* 2001). On the other hand, IL-4 appears to maintain the epithelial phenotype of hOSE, probably representing integrity of hOSE layer after repair. Co-treatment of hOSE cells with both cytokines resulted in a mixture of epithelio-fibroblast-like cells, implying that a probable role of IL-4 is to alleviate IL-1α-related inflammation. To further investigate this hypothesis, it would be interesting to quantify relative levels of cytokeratin positive cells in response to treatments (control, IL-1α, IL-4, IL-1α+IL-4).

Regarding 3β-HSD1 and 3β-HSD2 mRNA expression levels, IL-4 appeared to override the effects of IL-1α. Strikingly, at the protein level, IL-1α and IL-4 co-treatment up-regulated total 3β-HSD protein relative to IL-4-induced 3β-HSD protein. This is implicative of a positive feedback loop mechanism where IL-4 anti-inflammatory action is further potentiated to restore IL-1α-induced wounding of hOSE.

None of the remaining pro-inflammatory (TNF-α, IL-6, GM-CSF and IL-18) and T lymphocyte-associated cytokines (IL-10) tested affected 3β-HSD1 or 3β-HSD2 mRNA, showing that the role of these cytokines in post-ovulatory wound healing of hOSE, if any, is other than controlling local bioavailability of progesterone and androgens. Surprisingly, nor TNF-α or IL-6 affected any of 3β-

HSD transcripts. We could expect all three cytokines to regulate similar genes, since TNF-α and IL-1α share common signalling properties and IL-6 secretion has been shown to be dependent on IL-1α action (Nash *et al.* 1999, Offner *et al.* 1995, Rae *et al.* 2004b, Verstrepen *et al.* 2008). However, an effect on downstream steroid signalling (*i.e.* at the receptor level) cannot be excluded. For example, IL-6/STAT-3 pathway is constitutively active in FSH-, LH- and oestrogen-stimulated hOSE and as such it deactivates PR, thereby abrogating progesterone-dependent signalling and promoting cytoproliferation (Mukherjee *et al.* 2005, Syed *et al.* 2002). Moreover, an effect of these two cytokines on 3β-HSD mRNA transcripts at earlier time-points cannot be excluded either, but as discussed above, restrictions on primary cell availability did not allow us to set up experiments with additional parameters.

In conclusion, in Chapter 4, we have demonstrated that both 3β-HSD1 and 3β-HSD2 mRNA are functional in hOSE cells. Both transcripts appear to be under inflammatory control during post-ovulatory wound healing processes. Opposite effects of IL-1α and IL-4 on the same target genes is an excellent paradigm of the tuning between inflammatory and anti-inflammatory activities of cytokines to restore homeostasis of a tissue. IL-1α differential regulation of 3β-HSD mRNA transcripts appears to monitor local steroid bioavailability during ovulation-associated inflammatory wounding of hOSE, whilst IL-4 responses appear to tightly control cytoproliferation of only genetically integral hOSE cells during post-ovulatory repair. The proposed model is given in Fig. 4.13.

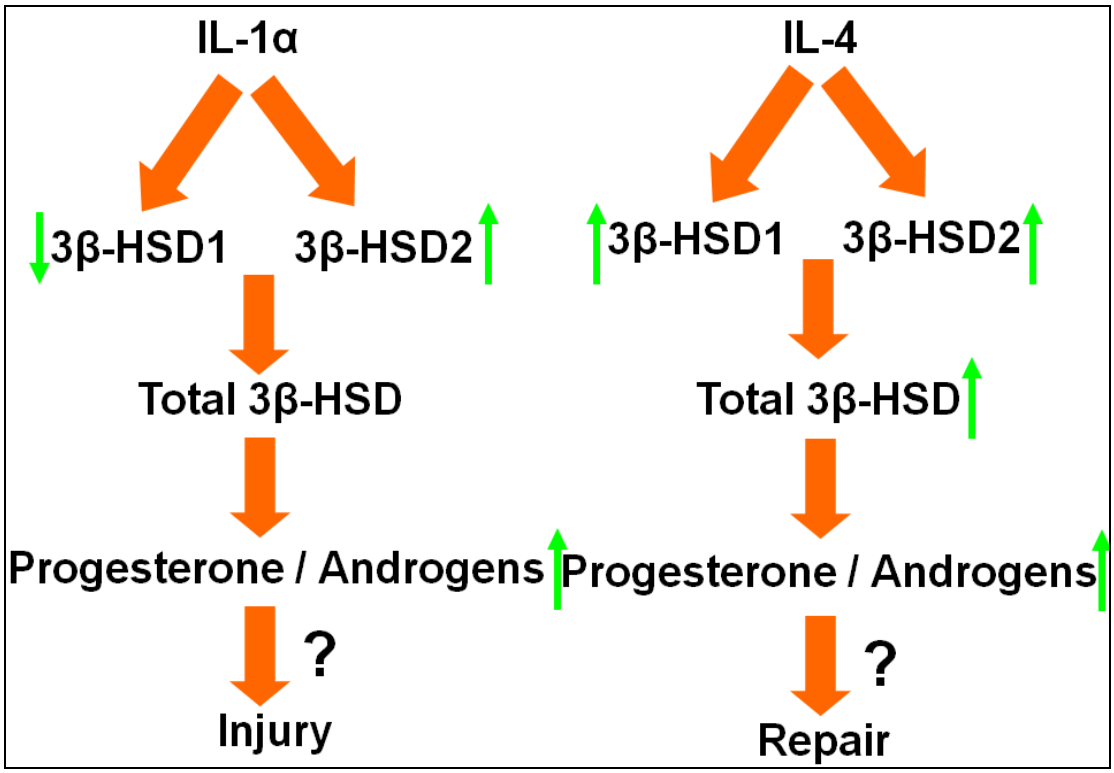


Figure 4.13: Role of IL-1α and IL-4 in post-ovulatory injury and repair of hOSE cells. This diagram briefly illustrates potential roles of IL-1α and IL-4 in ovulation-associated regeneration of hOSE cells. Ovulation associated wounding leads to reduced steroid biosynthesis as a result of IL-1α-attenuated 3β-HSD1 that is counteracted by IL-1α-induced 3β-HSD2. On the other hand, IL-4-induced 3β-HSD and therefore elevated steroid biosynthesis controls integral post-ovulatory repair of hOSE. Absence of arrow means no significance.

Chapter 5

Signalling transduction of 3β-HSD1 and 3β-HSD2 mRNA by IL-1α and IL-4

5.1 Introduction

In Chapter 4 it was demonstrated that 3β-HSD in the human ovarian surface epithelium is under inflammatory control during ovarian post-ovulatory injury and repair with 3β-HSD1 and 3β-HSD2 mRNA being differentially regulated by the pro-inflammatory IL-1α and T lymphocyte-associated IL-4. As described in Chapter 1, IL-1α action is mediated through transactivation of the IL-1 receptor type 1 (IL-1R1), whereas IL-4 action requires binding to the IL-4 receptor (IL-4R), both resulting in the initiation of cascades that control the regulation of IL-1α- and IL-4-responsive genes, respectively. In particular, IL-1α commonly activates the transduction of the classical inflammatory pathway, NF-κB as well as the MAPK pathways with SAPK/JNK and p38 MAPK the most common. On the other hand, IL-4R transactivation can result in the activation of PI-3K and ERK1/2 pathways, both mediating the cell cycle, as well as the STAT-6 pathway that is mainly involved in gene transcription. However, all three pathways can be parallel or complementary and may cooperate to mediate cell responses, from cell survival to gene expression. The proper response at every moment of the cell life is critical for the fate of the cell. The transcriptional regulation of genes that fulfil these services are under the control of a complex machinery that differs between different cell types at various stages of the cell cycle.

It was established in Chapter 4 that IL-1α suppressed 3β-HSD1 mRNA levels, whilst it stimulated 3β-HSD2 mRNA levels. Opposite activities of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA species suggests that IL-1α exerts its effects through activation of distinct pathways. Moreover, it was demonstrated that IL-4 induced both 3β-HSD1 mRNA and 3β-HSD2 mRNA, attesting to a role of IL-4 in the alleviation of inflammation and restoration of post-ovulatory stigma. Collectively, the aim of the present studies is to investigate the signalling transduction pathways that are potentially part of the network that regulates IL-1α- and IL-4-mediated 3β-HSD1 and 3β-HSD2 mRNA expression.

5.2 Subjects and Methods

As referred to above, IL-1α mainly exerts its effects through the NF-κB pathway and MAPK family. On the other hand, IL-4 generally acts through activation of the PI-3K, ERK1/2 MAPK and STAT-6 signalling pathways. Therefore, in order to elucidate the pathways through which IL-1α and IL-4 mediate 3β-HSD1 and 3β-HSD2, we treated primary culture hOSE cells with specific inhibitors for each of several pathways in the presence or absence of IL-1α or IL-4 (0.5ng/mL). Following incubation for 48h, cell harvest, RNA purification and RNA quantification/quality analysis were performed as described in Chapter 2. Then, RNA extracts where submitted to reverse-transcription and Taqman quantitative PCR to examine relative mRNA alterations among control, cytokine-treated and cytokine plus inhibitor-treated samples. Specific inhibitors along with their target pathways are depicted in Table 5.1. The basic clinical profiles of patients used for these studies are given in Table 5.2.

Table 5.1 Specific inhibitors used to suppress target pathways

Inhibitor	Target molecule	Target Pathway	Concentration	Reference
PD98059	MEK1/2	ERK1/2	50μM	(Moon *et al.* 2007)
SB203580	p38 protein (ATP-pocket)	p38 MAPK	10μM	(Bazuine *et al.* 2005)
BAY117082	IκB	NF-κB	1μM	(Skurk *et al.* 2004)
LY294002	p110 subunit	PI-3K	10μM	(Vlahos *et al.* 1994)
Leflunomide	Stat-6 (pyrimidine)	STAT-6	100μM	(Akiho *et al.* 2005)

All pathway inhibitors used were purchased from Merck Biosciences (Beeston, Nottingham, UK) and were diluted in DMSO. Untreated control cells were treated with empty vehicle (DMSO)

Table 5.2 Clinical picture of patients used for hOSE inhibitor studies

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
38	7332	1998/6/33	42	TAHBSO	Prophylactic	(21) Luteal	BAY11
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Follicular	BAY11
39	7336	04/S1103/36	40	TAH	Prophylactic	(22) Luteal	BAY11
40	7345	04/S1103/36	28	DiagLapar	Pain	(9) Follicular	BAY11
41	7320	04/S1103/36	47	TAHBSO	HMB	n/s	SB20
38	7332	1998/6/33	42	TAHBSO	Prophylactic	(21) Luteal	SB20
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Follicular	SB20
39	7336	04/S1103/36	40	TAH	Prophylactic	(22) Luteal	SB20
42	7354	04/S1103/36	46	TAH	HMB	n/s	PD98
43	7348	04/S1103/36	40	TAH	Fibroids	n/s	PD98
44	9018	04/S1103/36	33	DiagLapar	Mid-cycle pain	(19) Luteal	PD98
44	9018	04/S1103/36	33	DiagLapar	Mid-cycle pain	(19) Luteal	LY29
45	5517	04/S1103/36	43	LapSter	Fibroids	(21) Luteal	LY29
10	5536	04/S1103/36	23	DiagLapar	Dysmenorrhoea	(4) Follicular	LY29
11	5537	1998/6/33	24	DiagLapar	Dysmenorrhoea	(28) Luteal	LEF
12	7383	04/S1103/36	43	TAHBSO	Fibroids	(13) Luteal	LEF
37	7414	04/S1103/36	22	DiagLapar	Pelvic Pain	(9) Follicular	LEF

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. BAY11: BAY117082, SB20: SB203580, PD98: PD98059, LY29: LY294002, LEF: Leflunomide

5.3 Results

5.3.1 Involvement of MAPK signalling transduction pathways in IL-1α- and IL-4-mediated 3β-HSD1 and 3β-HSD2 mRNA expression

5.3.1.1 The role of the ERK1/2 signalling pathway in IL-1α and IL-4 mediation of 3β-HSD mRNA expression

In order to examine if IL-1α and IL-4 exert their effects on 3β-HSD1 and 3β-HSD2 mRNA through activation of an ERK1/2-related pathway, hOSE cells were treated *in vitro* with 50μM of PD98059, a selective inhibitor for this pathway, in the presence or absence of 0.5ng/mL of IL-1α or IL-4 for 48h. Suppression of ERK1/2 pathway did not affect IL-1α-mediated (b=p<0.05) or IL-4-elevated 3β-HSD1 and 3β-HSD2 mRNA levels (c=p<0.001) (Fig. 5.1, upper and lower panels, respectively).

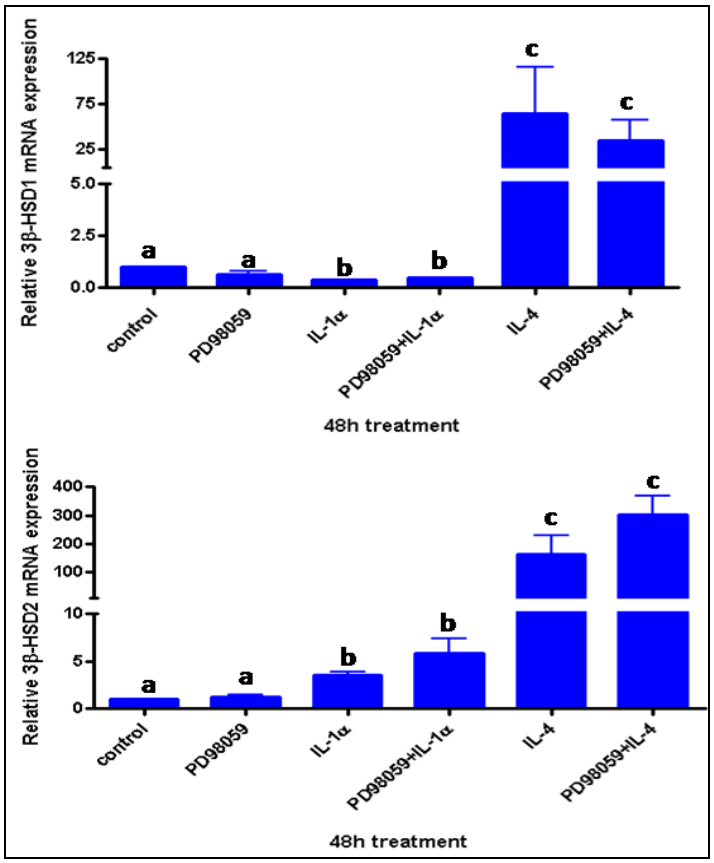


Figure 5.1: The role of the ERK1/2 signalling pathway in 3β-HSD mRNA expression. PD98959 (50μM) was added in IL-1α- or IL-4-treated hOSE cells for 48 hours (n=3, b=p<0.05, c=p<0.001).

5.3.1.2 The role of the p38 MAPK signalling pathway in IL-1α and IL-4 mediation of 3β-HSD mRNA species

Investigation of the potential involvement of a p38 MAPK signalling pathway in 3β-HSD1 and 3β-HSD2 transcriptional regulation was assessed by suppression of this pathway with SB203580 (10μM), a selective inhibitor of this pathway. Intriguingly, addition of the inhibitor to hOSE cells significantly reduced basal 3β-HSD1 and 3β-HSD2 mRNA levels (Fig. 5.2, upper and lower panel respectively, b=$p<0.05$). However, it did not further inhibit IL-1α-decreased 3β-HSD1 mRNA levels and it did not affect the IL-4-increased levels of 3β-HSD1 mRNA (Fig. 5.2, upper panel; n=4, b=$p<0.05$, c=$p<0.001$). On the other hand, induction of 3β-HSD2 mRNA levels by IL-1α and IL-4 (0.5ng/mL) for 48h were completely blocked when SB203580 was added for 48h (Fig. 5.2, lower panel; n=4, c=$p<0.05$, d=$p<0.001$).

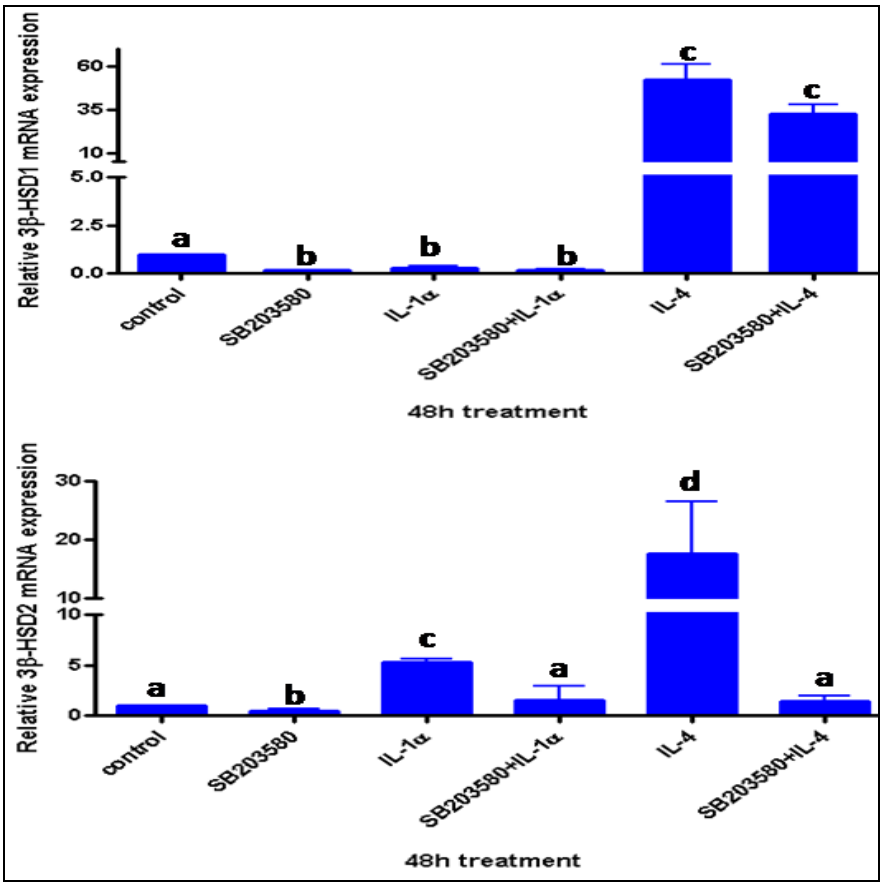


Figure 5.2: The role of the p38 MAPK signalling pathway in 3β-HSD mRNA expression. SB203580 (10μM) was added in IL-1α- or IL-4-treated hOSE cells (0.5ng/mL) for 48 hours. qPCR was performed to investigate the effects of the inhibitor alone or in the presence of IL-1α and IL-4 on the levels of 3β-HSD1 and 3β-HSD2 mRNA (upper panel, n=4, b=$p<0.05$, c=$p<0.001$; lower panel, n=4, b,c=$p<0.05$, d=$p<0.001$).

5.3.2 Involvement of the NF-κB signalling transduction pathway in IL-1α- and IL-4-mediated 3β-HSD1 and 3β-HSD2 mRNA expression

Considering that inflammatory responses are commonly mediated by NF-κB-associated inflammatory pathways, we suppressed NF-κB signalling pathways by addition of BAY117082 (1μM) for 48h and tested IL-1α and IL-4 (0.5ng/mL) effects on the transcriptional regulation of 3β-HSD1 and 3β-HSD2 mRNA levels (Fig. 5.3, upper and lower panel, respectively). Abrogation of 3β-HSD1 mRNA levels by IL-1α was blocked in the presence of BAY117082, whilst IL-4-induced 3β-HSD1 mRNA levels were not affected (Fig. 5.3, upper panel; n=4, b=$p<0.05$, c=$p<0.001$). Similarly, IL-1α-elevated 3β-HSD2 mRNA levels were suppressed on the addition of the inhibitor, but no influence on the IL-4 stimulatory effect on 3β-HSD2 mRNA was observed (Fig. 5.3, lower panel; n=4, b=$p<0.05$, c=$p<0.001$).

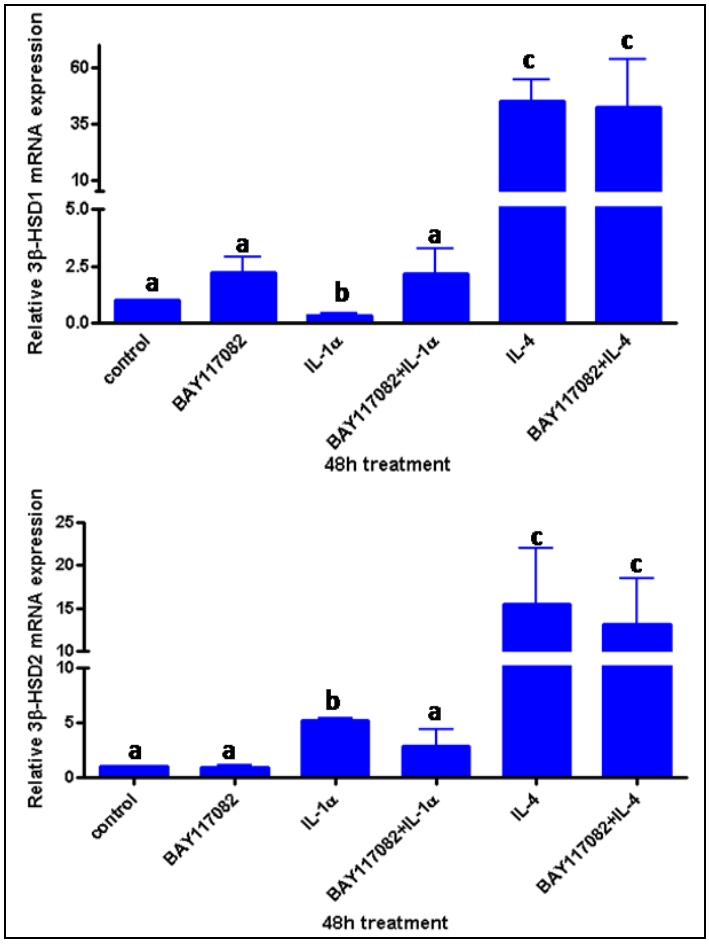


Figure 5.3: The role of the NF-κB signalling pathway in 3β-HSD mRNA expression. hOSE cells collected from 4 distinct patients were treated with 0.5ng/mL of IL-1α or IL-4 in the presence or absence of 1μM BAY117082 for 48h to inhibit NF-κB signalling pathway (n=4, b=$p<0.05$, c=$p<0.001$).

5.3.3 Involvement of the PI-3K signalling transduction pathway in IL-1α- and IL-4-mediated effects on 3β-HSD1 and 3β-HSD2 mRNA expression

We next tested if IL-1α and/or IL-4 action utilised the PI-3K signalling transduction pathway to exert their effects on the transcriptional regulation of 3β-HSD1 and 3β-HSD2 mRNA. Therefore, we suppressed the PI-3K signalling pathway with the selective inhibitor, LY294002 (10μM) (Fig. 5.4). Stimulatory effects of IL-4 (0.5ng/mL) on 3β-HSD1 and 3β-HSD2 mRNA levels were attenuated (Fig. 5.4 upper and lower panel, respectively; n=3, c=$p<0.001$, d=$p<0.01$). Moreover, *in vitro* addition of LY294002 completely blocked IL-1α-increased 3β-HSD2 mRNA (Fig. 5.4, lower panel; n=3, b=$p<0.05$).

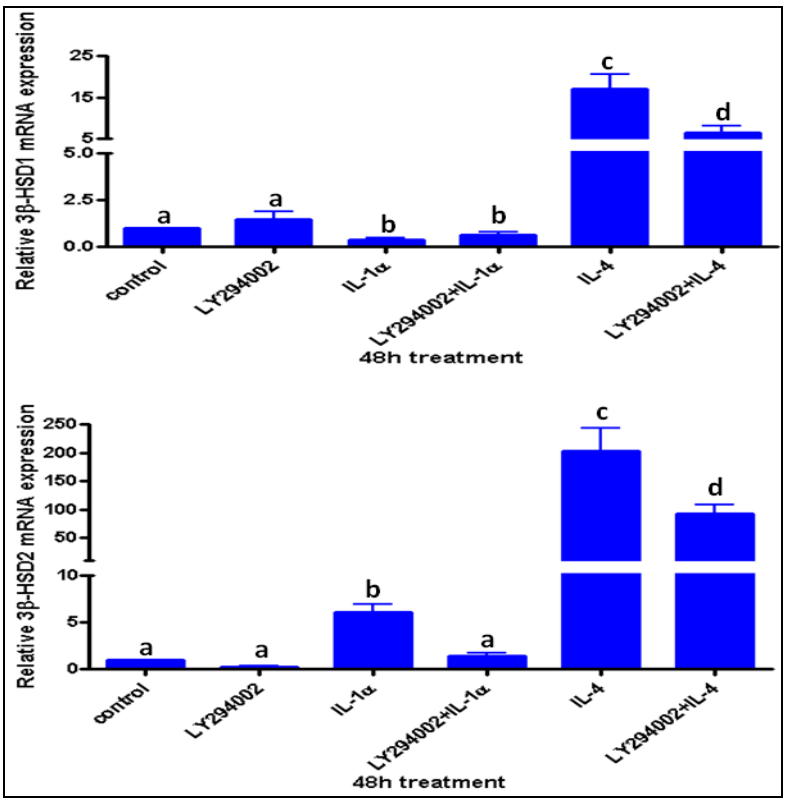


Figure 5.4: The role of the PI-3K signalling pathway in 3β-HSD mRNA expression. LY294002 (10μM) was added in IL-1α- or IL-4-treated hOSE cells (0.5ng/mL) for 48h. Effects of the inhibitor alone or in the presence of the cytokine on the levels of 3β-HSD1 and 3β-HSD2 mRNA was studied by Taqman qPCR (n=3, b=$p<0.05$, c=$p<0.001$, d=$p<0.01$).

5.3.4 Involvement of the STAT-6 signalling transduction pathway in IL-1α- and IL-4-mediated effects on 3β-HSD1 and 3β-HSD2 mRNA expression

An additional candidate pathway that could be part of the signalling cascades initiated by IL-1α and/or IL-4 was STAT-6. As such, we suppreseed the STAT-6 pathway *in vitro* by treatment of hOSE cells with the STAT-6 inhibitor, leflunomide (100μM), in the presence or absence of IL-1α or IL-4 (0.5ng/mL). Following incubation with those cytokines for 48 hours and RNA preparation, RNA samples were submitted to reverse transcription and Taqman Real-Time PCR to assess mRNA levels of 3β-HSD1 and 3β-HSD2 (Fig. 5.5 upper and lower panels, respectively). Stimulatory effects of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA levels were significantly reduced. IL-1α responses were not affected (Fig. 5.5 upper and lower panels, respectively; n=3, b=p<0.05, c=p<0.001, d=p<0.01).

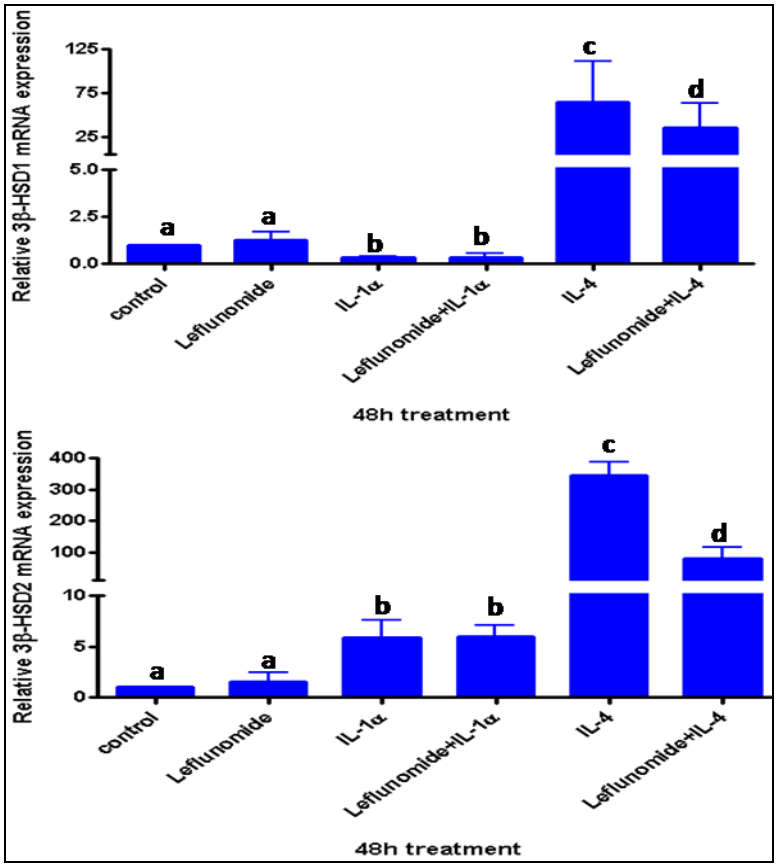


Figure 5.5: The role of the STAT-6 signalling pathway in 3β-HSD mRNA expression. Leflunomide (100μM) was added in IL-1α- or IL-4-treated hOSE cells (0.5ng/mL) for 48h and effects of 3β-HSD1 and 3β-HSD2 mRNA levels were studied (n=3, b=p<0.05, c=p<0.001, d=p<0.01).

5.3.5 Effects of IL-1α and IL-4 on IL-1 receptor type 1 and IL-4 receptor mRNA levels

Following establishment of the signalling transduction pathways that potentially relate to IL-1α and IL-4 action in 3β-HSD1 and 3β-HSD2 transcriptional regulation, we then explored if there was any cross-reaction of IL-1α or IL-4 in the expression of IL-1 receptor type 1 (IL-1R1) and IL-4 receptor (IL-4R), respectively. IL-1α (0.5ng/mL) did not affect IL-4R mRNA levels (Fig. 5.6, lower panel) while IL-4 (0.5ng/mL) also did not impact upon IL-1R1 mRNA levels (Fig. 5.6, upper panel).

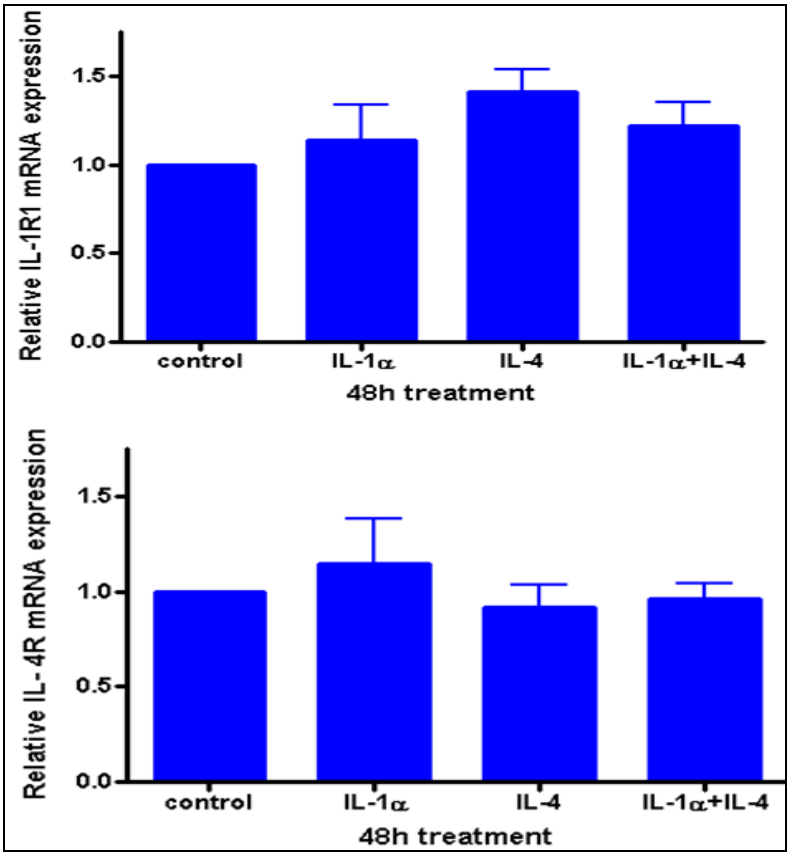


Figure 5.6: Effects of IL-1α and IL-4 on IL-1R1 and IL-4R mRNA expression. Combined data of 3 independent experiments. IL-1α and IL-4-treated hOSE cells (0.5ng/mL) were evaluated for IL-1R1 and IL-4R mRNA expression levels (n=3, upper panel and lower panel, respectively).

5.3.6 Control experiment: Effects of the various inhibitors employed on the IL-1R1 and IL-4R mRNA levels

To ascertain specificity and sensitivity of the inhibitors for components downstream of receptor transactivation, we examined whether the various selective inhibitors used affected IL-1R1 and IL-4R mRNA. Experiments with 3 separate hOSE samples showed that none of the inhibitors assayed affected either IL-1R (Fig. 5.7, upper panel) or IL-4R mRNA (Fig. 5.7, lower panel).

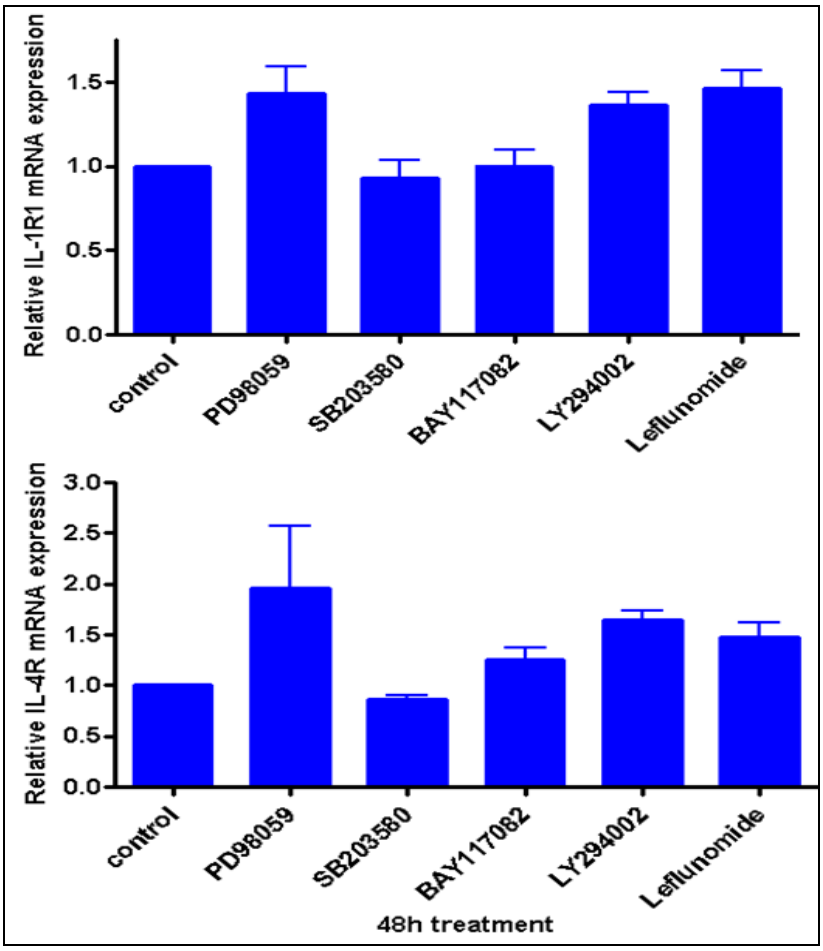


Figure 5.7: Effects of pathway inhibitors on IL-1R1 and IL-4R mRNA levels. Combined experiments of 3 distinct hOSE samples. The doses of the inhibitors used to elucidate mechanisms through which cytokines affect 3β-HSD1 and 3β-HSD2 mRNA were applied and effects on IL-1R1 (upper panel) or IL-4R (lower panel) mRNA were tested (n=3).

5.3.7 Summary of pathway inhibitor experiments

Table 5.3 Effects of inhibitors on transcriptional regulation of target genes

Treatment	Target Pathway	Transcriptional regulation of target genes			
		3β-HSD1	**3β-HSD2**	**IL-1R1**	**IL-4R**
PD98059	ERK1/2	n/s	n/s	n/s	n/s
SB203580	p38 MAPK	↓	↓	n/s	n/s
BAY117082	NF-κB	n/s	n/s	n/s	n/s
LY294002	PI-3K	n/s	n/s	n/s	n/s
Leflunomide	STAT-6	n/s	n/s	n/s	n/s
IL-1α		↓	↑	n/s	n/s
PD98059+IL-1α	ERK1/2	↓	↑	-	-
SB203580+IL-1α	p38 MAPK	↓	n/s	-	-
BAY117082+IL-1α	NF-κB	n/s	n/s	-	-
LY294002+IL-1α	PI-3K	↓	↑	-	-
Leflunomide+IL-1α	STAT-6	↓	n/s	-	-
IL-4		↑	↑	n/s	n/s
PD98059+IL-4	ERK1/2	↑	↑	-	-
SB203580+IL-4	p38 MAPK	↑	n/s	-	-
BAY117082+IL-4	NF-κB	↑	↑	-	-
LY294002+IL-4	PI-3K	↑ **(**Partially reversed IL-4 effect)	↑ **(**Partially reversed IL-4 effect)	-	-
Leflunomide+IL-4	STAT-6	↑ **(**Partially reversed IL-4 effect)	↑ **(**Partially reversed IL-4 effect)	-	-

↓: down-regulation, ↑: up-regulation, n/s: not significant, -: not tested

5.4 Discussion

In Chapter 4, we showed that IL-1α differentially regulated 3β-HSD1 and 3β-HSD2 mRNA in primary cultured hOSE cells, resulting in a balance of steroid biosynthesis as suggested by 3β-HSD protein and activity studies. In this Chapter, we show that IL-1α utilises a number of pathways to fulfil these opposing effects on the two isoforms. IL-1α commonly uses NF-κB pathways for both transcripts, however p38 MAPK and PI-3K also appear to be required for IL-1α-increased 3β-HSD2 mRNA expression. Moreover, we demonstrate that IL-4-induced 3β-HSD1 and 3β-HSD2 mRNAs in post-ovulatory repair are mediated by PI-3K and STAT-6 pathways. Also, at the case of 3β-HSD2 mRNA, a cross-talk of these two signalling pathways with p38 MAPK appears essential for this effect.

The involvement of p38 MAPK in IL-1α and IL-4 activities in 3β-HSD1 and 3β-HSD2 mRNA was studied through inhibition of this pathway with the pyridinyl imidazole compound, SB203580. This inhibitor specifically interacts with the ATP pocket of the p38 MAPK protein, thereby suppressing its action (Tong *et al.* 1997). Inhibition of the p38 MAPK pathway resulted in a blocking of IL-1α- and IL-4-induced 3β-HSD2 mRNA suggesting a role for this pathway in cytokine responses. Intriguingly, the inhibitor alone down-regulated basal 3β-HSD1 and 3β-HSD2 mRNA levels, suggesting that the regulation of these genes is dependent on this pathway. Involvement of the p38 MAPK signalling pathway in the transcriptional regulation of 3β-HSD is a novel finding in the documented record of 3β-HSD regulation in a variety of tissues (see Chapter 1, Section *1.4.2.2*). However, this effect is consistent with p38 MAPK involvement in cell death and cell apoptosis (Xia *et al.* 1995). Although IL-1α is a strong activator of p38 MAPK in several cell systems, there is no evidence that it directly induces cell apoptosis, at least *in vitro* (Xia *et al.* 1995), suggesting that the p38 MAPK pro-apoptotic effects are probably exerted indirectly. Our data suggest that, at least in hOSE cells, IL-1α-induced p38 MAPK triggers apoptotic effects through induction of transcriptional activity of apoptotic-associated genes such as 3β-HSD1 and 3β-HSD2 mRNA. Physiologically,

follicular rupture is followed by sloughing of the ovarian cell surface, a process that is considered to be mediated by apoptosis (Murdoch 1995). Accordingly, in a number of studies that were acknowledged in Chapter 1 (Section 1.5), most of the IL-1-regulated genes that involve p38 MAPK are proteolytic (*e.g.* t-PA), ECM-related (*e.g.* gelatinase) or pro-inflammatory (*e.g.* COX-2). Intriguingly, all of these genes have been reported to be mediated in post-ovulatory inflammation and tissue remodelling of hOSE (Gubbay *et al.* 2005, Murdoch 1999, Rae *et al.* 2004a, Yang *et al.* 2004). Besides the pro-inflammatory-related genes mediated by IL-1α-induced p38 MAPK, herein we now show that transcriptional regulation of anti-inflammatory and apoptotic genes such as 3β-HSD is also possible, potentially reflecting a negative feedback loop mechanism of IL-1α action through which hOSE recovers from tissue damage. Further support of this concept is the fact that IL-1α massively up-regulates 11β-HSD1 mRNA and activity in hOSE, thereby sustaining local anti-inflammatory glucocorticoid regeneration to counteract hOSE post-ovulatory damage (Yong *et al.* 2002). Remarkably, IL-1α-induction of 11β-HSD1 mRNA is reversed by SB203580, a p38 MAPK inhibitor (Rae M.T., unpublished observations). Moreover, TAK-1 that is upstream of p38 MAPK and is induced by IL-1R1 has been reported to be stimulated in response to TGF-β treatment in mink lung epithelial and murine osteoblastic cells (Yamaguchi *et al.* 1995). It is notable that TGF-β has been established to have an apoptotic role in hOSE (Berchuck *et al.* 1992, Choi *et al.* 2001c, Evangelou *et al.* 2000). Moreover, TGF-β1 has been shown to highly up-regulate 3β-HSD1 in OSE-C2 cells, an immortalised non-malignant hOSE cell line (Papacleovoulou, unpublished observations). A relevant anti-inflammatory and protective role of p38 MAPK pathway has been also established in prostate cancer. Blockage of this pathway through over-expression of the MKP-1 phosphatase or addition of the SB203580 inhibitor rescued cells from cell death (Wu & Bennett 2005).

Regarding IL-4-induced 3β-HSD1 and 3β-HSD2 mRNA, the p38 MAPK pathway appeared to be indispensable for 3β-HSD2 transcriptional activity but not for 3β-HSD1 mRNA expression, indicative that the same signal can result in

differential regulation of target genes. This concordance with the studies on IL-1α-induced 3β-HSD2 mRNA indicates that transcriptional regulation of this gene involves p38 MAPK action. Interestingly, p38 MAPK is not notable as a universal signalling pathway for IL-4 action but is cell type-dependent (Hunt *et al.* 2002). For instance, IL-4 can stimulate the p38 MAPK signalling pathway in murine T and B lymphocyte cell lines, CT6 and BA/F3 respectively, but not in a murine macrophage cell line (Hunt *et al.* 2002). Moreover, IL-4-induced TIMP-2, an anti-inflammatory response, has been reported to be mediated by p38 MAPK activation as shown in human dermal fibroblasts (Ihn *et al.* 2002). Additionally, the suppressor of the cytokine signalling-3 (SOCS-3) has been demonstrated to be triggered by IL-4-induced p38 MAPK in murine splenic cells (Canfield *et al.* 2005).

Collectively, it appears that anti-inflammatory responses of IL-4 and IL-1α to 3β-HSD2 mRNA in hOSE are mediated by the stimulation of the p38 MAPK signalling pathway. The same role could be also fulfilled by the other stress-induced pathway, the JNK signalling pathway. However, we were unable to study potential involvement of this kinase due to the poor specificity of the inhibitors available for this pathway (data not shown). Additional reports that question the specificity of these inhibitors have been previously described (Bain *et al.* 2003, Wu & Bennett 2005).

The effects of the cytokines on 3β-HSD1 and 3β-HSD2 mRNA did not appear to involve ERK1/2 signalling pathways as the specific inhibitor for the ERK1/2 activator, MEK1/2, did not alter IL-1α or IL-4 activities in 3β-HSD1 and 3β-HSD2 mRNA levels. However, these responses are in accordance with the mitogenic role of this group of MAPKs along with the proposed anti-inflammatory and pro-apoptotic roles that 3β-HSD1 and 3β-HSD2 mRNA have at least in hOSE. On the other hand, ERK1/2 activation by FSH and HGF to trigger cytoproliferation of hOSE has been described (Choi *et al.* 2002, Gubbay *et al.* 2004).

Along with participation of p38 MAPK in mRNA transcriptional regulation of 3β-HSDs, NF-κB was also involved in IL-1α-mediated 3β-HSD1 and 3β-HSD2

mRNA responses. As mentioned in Chapter 1 (Section 1.5), NF-κB signalling is an indispensable component of the TIR superfamily, through which IL-1α exerts its actions. Remarkably, whereas most of the genes regulated by NF-κB are pro-inflammatory, we are the first to show that an NF-κB pathway is involved in transduction of 3β-HSD1 and 3β-HSD2 mRNA by cytokines in hOSE cells as revealed by blockage of IL-1α-mediated 3β-HSD mRNA species after treatment with BAY117082 that specifically abolishes phosphorylation of IκB and hence translocation of NF-κB into nucleus. The involvement of this pathway in pro-inflammatory IL-1α effects on 3β-HSD1 is consistent with the nature of NF-κB responses. However, and somehow surprisingly, this pathway also appears to be involved in an anti-inflammatory effect such as the stimulation of 3β-HSD2 mRNA by IL-1α. A negative feedback loop mechanism could be behind this effect. It can be speculated that IL-1α-activated NF-κB to induce 3β-HSD2 and thus progesterone formation and action through PR could in turn abolish pro-inflammatory NF-κB responses. In support of this, it has been proposed previously that NF-κB and PR mutually suppress each other's activity (van der Burg & der Saag 1996). Precisely, it has been demonstrated that progesterone's immunosuppressive and anti-inflammatory action is subject to its ability to harness the initiation of inflammatory processes such as ovulation through inhibition of NF-κB signalling. For instance, in endometrium it has been found that progesterone through nuclear PR impacts upon anti-apoptotic NF-κB through driving transcription of genes that inhibit NF-κB and suppression of genes that activate NF-κB (Davies *et al.* 2004). On the other hand, NF-κB activation abrogates PR-associated action, thereby promoting pro-inflammatory responses (Kalkhoven *et al.* 1996). Our data suggest that these effects might well be mediated by 3β-HSD regulation that essentially controls local bioavailability of the active ligand and access to PR, a crucial event for transactivation of downstream progesterone signalling.

IL-4-induced 3β-HSD1 and 3β-HSD2 mRNA levels were not affected by the presence of BAY117082, implying that IL-4 activity is not controlled by this pathway. On the other hand, another possible explanation could be that IL-4 exerts

its anti-inflammatory effects through antagonising IL-1α-induced pro-inflammatory effects. This appears to be the case in IL-1α plus IL-4 co-treatment studies described in Chapter 4 (Section 4.3.5). If this is the case, it is interesting that these antagonistic effects appear to be mediated downstream of IL-1R and IL-4R transactivation, as neither IL-1α nor IL-4 altered IL-4R or IL-1R mRNA. As such, it could be speculated that IL-4 anti-inflammatory activities are achieved through inhibition of the pro-inflammatory NF-κB responses and activation of pro-apoptotic p38 MAPK pathway. In support of this, antagonistic effects between NF-κB and p38 MAPK have been demonstrated. Precisely, in primary embryonic fibroblasts of a mouse model, it has been illustrated that inhibition of anti-apoptotic NF-κB pathway results in increased JNK and p38 MAPK activities (Garcia-Cao *et al.* 2003). Nevertheless, a further complexity of this issue is that NF-κB transcription factor, as described earlier, was shown to be involved in the IL-1α-induced 3β-HSD2 mRNA action, an anti-inflammatory effect, possibly reflecting a negative feedback loop mechanism. Another or a complementary explanation for this anti-inflammatory response could be a cross-talk mechanism of NF-κB with the PI-3K and the p38 MAPK-induced pathways (see below).

IL-1α-induced 3β-HSD2 mRNA levels were also found to be a result of activation of the PI-3K signalling pathway. Although the PI-3K pathway is not the most common target of IL-1R1 transactivation, recent reports suggest involvement of this pathway in IL-1 signalling cascades. A role of this pathway in IL-1β-mediated metabolic pathways (*i.e.* lactate production) has been reported in rat Sertoli cells (Riera *et al.* 2007). Moreover, this pathway has been shown to be an intermediate of IL-1-regulated NF-κB transcription factor action, mediating IL-8 mRNA and secretion in human glioblastoma cells (Funakoshi *et al.* 2001). Importantly, in the HepG2 cell line, PI-3K appears to trigger NF-κB transcriptional activity through p65 phosphorylation (Sizemore *et al.* 1999), consistent with the concept that IκB phosphorylation and ubiquitination are not sufficient for NF-κB activation and nuclear translocation (Zhong *et al.* 1997). Moreover, it has been also found that both p38 MAPK and PI-3K signalling pathways co-operate in IL-1-induced AP-1

transcriptional activity in glioblastoma cells (Funakoshi *et al.* 2001). Additionally, t-PA activity in osteosarcoma cells appears to be dependent on PI-3K and p38 MAPK activation (Chang *et al.* 2006). Jointly, these findings suggest that PI-3K could be an intermediate activator of the p38 MAPK- and NF-κB-dependent 3β-HSD2 mRNA expression. Therefore, it is intriguing that IL-1α utilises different signalling pathways to regulate different genes, and this is probably part of the opposite effects that this proxy exerts on the two 3β-HSD mRNA species.

Additionally, the PI-3K pathway appeared a component in the effects of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA species as these effects were partially but significantly suppressed when LY294002, a specific inhibitor of PI-3K activity, was added. As noted in Chapter 1 (Section 1.5), this pathway is mainly involved in mitogenic activities of IL-4, since it usually activates the Akt proto-oncogene that sustains cell proliferation and survival and blocks apoptosis (Franke *et al.* 1997). As discussed in Chapter 4, IL-4 is mainly secreted during the luteal phase of the menstrual cycle when ovarian tissue remodelling and repair of the stigma take place, suggesting that IL-4 promotes hOSE cell proliferation to heal the wound. Physiologically, IL-4-stimulated 3β-HSDs through PI-3K activation to induce local generation of progesterone in hOSE might be a mechanism through which IL-4 monitors controlled proliferation of only integral hOSE cells. As such, genetically damaged cells undergo progesterone-associated apoptosis during post-ovulatory repair. Consistent with this model, it has been previously shown that IL-4-treated porcine endothelial cells are protected from TNF-α-induced injury through activation of PI-3K-associated cell survival responses (Grehan *et al.* 2005). Furthermore, in breast cancer cell lines PI-3K signalling pathways were demonstrated to be an intermediate component of IL-4-induced 3β-HSD1 activity (Gingras *et al.* 2000). On the other hand, impaired PI-3K signalling at post-ovulatory repair might lead to dysfunction of related transcriptional machinery. This could have profound implications in the aetiology of ovarian cancer as links between amplification of PI-3K components and EOC have been described (Mills *et al.* 2001, Shayesteh *et al.* 1999, Wong *et al.* 2001, Yuan *et al.* 2000).

The STAT-6 pathway is ubiquitously activated by IL-4 and is the major component of transcriptional regulation of IL-4-responsive genes. Leflunomide, a STAT-6 inhibitor, moderately though significantly suppressed IL-4-induced 3β-HSD1 and 3β-HSD2 mRNA levels. Accordingly, the promoter of the gene for 3β-HSD1 includes two STAT-6 recognition sites. Similarly, the gene for 3β-HSD2 also contains a putative STAT-6 recognition site in the promoter (Gingras *et al.* 2000). It is not clear from the present data if the multiple signalling pathways that participate in IL-4 responses are parallel or complementary. Thus, STAT-6 transactivation might involve PI-3K activity as seen in the case of 3β-HSD1 or even p38 MAPK as seen in the case of 3β-HSD2 mRNA. Indeed, it has been established that transactivation of STAT-6-induced transcriptional regulation of IL-4-responsive genes is mediated by p38 MAPK activity (Pesu *et al.* 2002). Besides, in the keratinocyte HaCaT cell line, it has been demonstrated that IL-4 exerts some of its anti-inflammatory effects through enhancement of the IL-13 receptor αII mRNA and protein (Th-2 response) and this is achieved through p38 MAPK activation that in turn facilitates IL-4-dependent STAT-6 transcriptional regulation (David *et al.* 2001). Moreover, albeit that involvement of PI-3K is mainly in cell cycle activities of IL-4, its participation in transcriptional regulation of IL-4-mediated genes has been also established (Gingras *et al.* 2000, Wang *et al.* 1997, Wang *et al.* 1998).

The participation of multiple signalling pathways in the regulation of 3β-HSD protein/activity cannot be predicted at the moment. It is widely accepted that protein kinases can impact upon transcriptional and/or post-transcriptional and/or translational and/or post-translational regulation of responsive genes. For example, in human fibroblasts, it has been shown that p38 MAPK regulates IL-6 production, but it does not affect IL-6 mRNA synthesis (Ridley *et al.* 1997).

In order to ascertain that the tested inhibitors as well as the doses used exclusively abolish target molecules of signalling pathways and all the effects obtained are not a consequence of cytokine receptor activation/suppression, we assessed the effects of inhibitors alone on the respective receptors. Basal levels of IL-

1R1 and IL-4R did not alter in response to any of the pathway inhibitors, suggestive of action of the latter in downstream of the receptors signalling molecules.

In conclusion, we have demonstrated that IL-1α utilises different panels of signalling molecules to mediate 3β-HSD1 and 3β-HSD2 mRNA transcription, probably explaining its opposing effects on these genes. Its pro-inflammatory effect on 3β-HSD1 is mediated by NF-κB, whereas the anti-inflammatory action in 3β-HSD2 is monitored by NF-κB, p38 MAPK and PI-3K signalling pathways. On the other hand, the IL-4 anti-inflammatory effects on hOSE during post-ovulatory repair are mediated through STAT-6 and PI-3K signalling networks. Moreover, in the case of 3β-HSD2, p38 MAPK is essential for IL-4 action and this implies that these two very similar genes are regulated by different mechanisms. A summary of our proposed model is presented in Fig. 5.8.

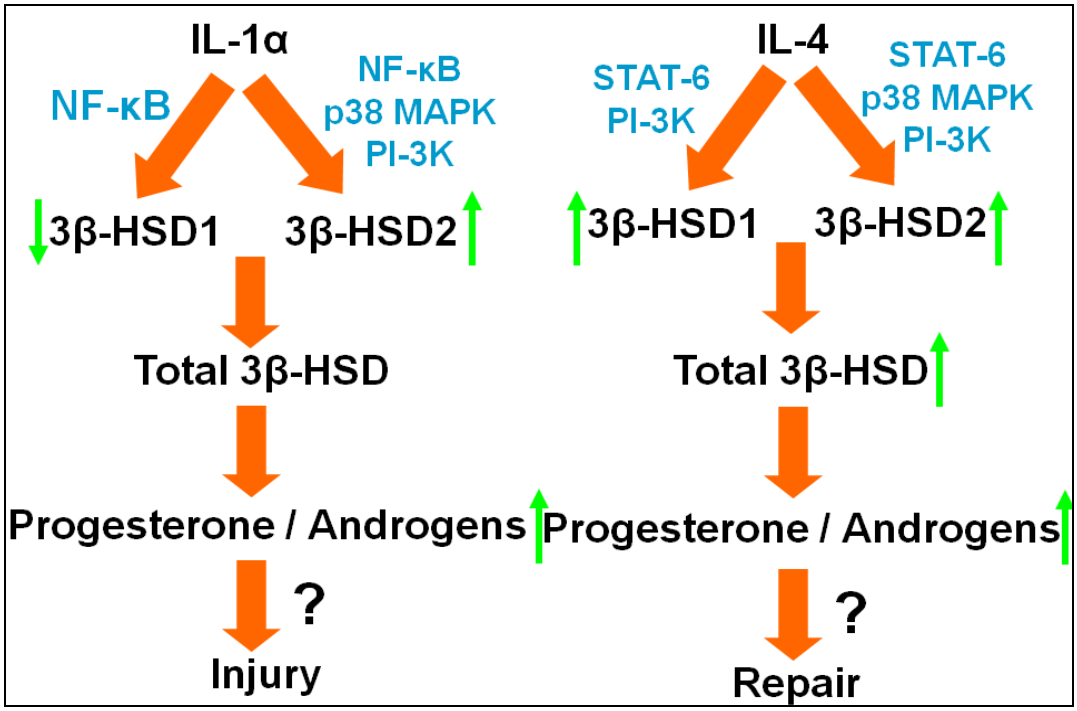


Figure 5.8: Signalling pathways involved in IL-1α and IL-4 responses to 3β-HSD1 and 3β-HSD2 mRNA. Schematic representation of the proposed model and mechanisms for the role of IL-1α and IL-4 in injury and repair of hOSE with respect to 3β-HSD involvement. Absence of arrow delineates no significance.

Chapter 6

Expression of androgen and progesterone receptors in the human ovarian surface epithelium: Regulation by IL-1α and IL-4

6.1 Introduction

In Chapters 3, 4 and 5, the focus was on the mechanisms that control progesterone and androgen pre-receptor metabolism and bioavailability in primary hOSE cells through investigation of the regulation of 3β-HSD1 mRNA and 3β-HSD2 mRNA expression along with total 3β-HSD protein and activity. However, local bioavailability of androgens and progestogens is not sufficient for their local action. As discussed in Chapter 1 (Section 1.4), the action of these two hormones is achieved through their binding to the cognate receptors, namely androgen (AR) and progesterone (PR) receptors. As described in Chapter 1 (Section 1.2.3), apart from the capacity of hOSE to form active steroid ligands, it has the ability to promote them through expression of nuclear steroid receptors. As such, the human ovarian surface expresses functional AR and PR, suggesting that the active products of the 3β-HSD enzyme, androstenedione and progesterone can directly exert their cytoproliferative and apoptotic/anti-inflammatory effects respectively in hOSE. So far, it has been established that 3β-HSD mRNA species are differentially regulated by IL-1α and IL-4, implying altered bioavailability of steroid ligands during post-ovulatory wounding and healing of the ovarian cell surface. Nonetheless, there is a lack of evidence whether these two cytokines could also affect downstream signalling of androgens and progestogens through regulation of the AR and PR during post-ovuatory injury and repair of hOSE.

Therefore, the aim of this Chapter is the investigation of the effects of IL-1α and IL-4 on AR and PR mRNA and protein expression in primary hOSE cells. After establishment of a potential role of any of these two cytokines in AR and PR expression, we further investigated the mechanisms through which these effects might be exerted, focusing on pathway inhibitor studies as described in Chapter 5.

6.2 Subjects and Methods

In order to investigate the effects of cytokines on AR and PR mRNA and protein expression, we first ascertained that AR and PR proteins were present in the ovarian surface epithelium. We achieved that through fluorescence immunohistochemistry as described in Chapter 2. Then, using sqPCR, we confirmed that AR and PR mRNA expression levels remain *in vitro* in primary hOSE cell cultures. Treatments with IL-1α and IL-4 were applied to cultured hOSE monolayers and then Taqman qPCR and western immunoblotting were performed to measure the effects of these cytokines on AR and PR mRNA and protein levels, respectively relative to untreated control samples. Further treatments with pathway inhibitors in the presence or absence of cytokines were also conducted to elucidate the mechanisms through which cytokines might exert their effects on AR or PR mRNA expression levels. The basic clinical profile of patients used to complete these studies is presented in Tables 6.1, 6.2 and 6.3.

Table 6.1 *In situ* studies for AR and PR immunodetection

Patient No	Age (yrs)	Reason for surgery	Phase of menstrual cycle	IF
1	29	Amenorrhoeic past 5yrs; cyclical pain	-	AR, PR
2	40	Dysmenorrhoea; constant bleeding	-	AR, PR
3	40	Constant bleeding for 7 months; endometriosis history	-	AR, PR

IF: immunofluorescence

Table 6.2 Clinical profile of patients whose hOSE used for i*n vitro* studies for AR and PR mRNA/protein regulation by IL-1α and IL-4 cytokines

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
10	5536	04/S1103/36	23	DiagLapar	HMB/pain	(4) n/s	sqPCR
11	5537	04/S1103/36	24	DiagLapar	Dysmenorrhoea	(28) Luteal	sqPCR
12	7383	04/S1103/36	43	TAHBSO	Fibroids	(13) n/s	sqPCR
13	7384	04/S1103/36	42	TAH	HMB	(23) Luteal	sqPCR
14	9014	04/S1103/36	32	DiagLapar	Pelvic pain	(6) n/s	sqPCR
21	7357	05/S1103/14	33	TAH	HMB	n/s	IL-1α (TR)
25	7304	04/S1103/36	34	DiagLapar	Dysmenorrhoea	(7) Follicular	IL-1α (TR)
26	7361	04/S1103/36	39	LapSter	Constant bleeding	n/s	IL-1α (TR)
27	9012	04/S1103/36	34	LapSter	Prophylactic	n/s	IL-1α (TR)
6	5484	05/S1103/14	32	TAH	Prolapse	(24) Luteal	IL-4 (TR)
7	5497	04/S1103/36	39	TAH	Pelvic pain	(10) n/s	IL-4 (TR)
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	IL-4 (TR)
38	7314	04/S1103/36	43	Oopherectomy	HMB	n/s	IL-1α+IL-4
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	IL-1α+IL-4
39	7326	04/S1103/36	43	TAH	Fibroids	(27) n/s	IL-1α+IL-4
12	7383	04/S1103/36	43	TAHBSO	Fibroids	(13) n/s	IL-1α+IL-4
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	(9) n/s	IL-1α+IL-4
20	7423	04/S1103/36	49	TAH	Fibroids	(30) Luteal	IL-4
35	7435	04/S1103/36	43	TAH	Dysmenorrhoea	(14) Luteal	IL-4
36	7437	04/S1103/36	32	DiagLapar	Pelvic pain	(1)Mens/Fol	IL-4
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	n/s	IL-4
7	5497	04/S1103/36	39	TAH	Dysmenorrhea	(10) n/s	IL-4 protein
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Luteal	IL-4 protein
46	7437	04/S1103/36	32	DiagLapar	Pelvic pain	n/s	IL-4 protein

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, LapSter: laparoscopic sterilisation, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. TR: time-response

Table 6.3 Clinical picture of patients used for pathway inhibitor studies

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
38	7332	1998/6/33	42	TAHBSO	Prophylactic	(21) Luteal	BAY11
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Follicular	BAY11
39	7336	04/S1103/36	40	TAH	Prophylactic	(22) Luteal	BAY11
40	7345	04/S1103/36	28	DiagLapar	Pain	(9) Follicular	BAY11
41	7320	04/S1103/36	47	TAHBSO	HMB	n/s	SB20
38	7332	1998/6/33	42	TAHBSO	Prophylactic	(21) Luteal	SB20
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Follicular	SB20
39	7336	04/S1103/36	40	TAH	Prophylactic	(22) Luteal	SB20
42	7354	04/S1103/36	46	TAH	HMB	n/s	PD98
43	7348	04/S1103/36	40	TAH	Fibroids	n/s	PD98
44	9018	04/S1103/36	33	DiagLapar	Mid-cycle pain	(19) Luteal	PD98
44	9018	04/S1103/36	33	DiagLapar	Mid-cycle pain	(19) Luteal	LY29
45	5517	04/S1103/36	43	LapSter	Fibroids	(21) Luteal	LY29
10	5536	04/S1103/36	23	DiagLapar	Dysmenorrhoea	(4) Follicular	LY29
11	5537	1998/6/33	24	DiagLapar	Dysmenorrhoea	(28) Luteal	LEF
12	7383	04/S1103/36	43	TAHBSO	Fibroids	(13) Luteal	LEF
37	7414	04/S1103/36	22	DiagLapar	Pelvic Pain	(9) Follicular	LEF

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. BAY11: BAY117082, SB20: SB203580, PD98: PD98059, LY29: LY294002, LEF: Leflunomide

6.3 Results

6.3.1 Expression of androgen receptor in the human ovarian surface epithelium

Fluorescence immunohistochemistry in whole ovarian tissue was performed to immunodetect AR in the ovarian cell surface. It was demonstrated that AR (green) was located in the nuclei of ovarian surface epithelial cells (Fig. 6.1A, b-c). An antral follicle was used as a positive control (Fig. 6.1A, e-f).

In order to ascertain that AR mRNA expression remains in the primary cultured hOSE cells, sqPCR analysis of five hOSE independent samples was performed. AR was expressed in five hOSE samples from five patients (Fig. 6.1B, upper gel). Lutein-granulosa cells were used as a positive control (Fig. 6.1B, upper gel). cDNA integrity/quality was assessed with GAPDH mRNA (Fig. 6.1B, lower gel).

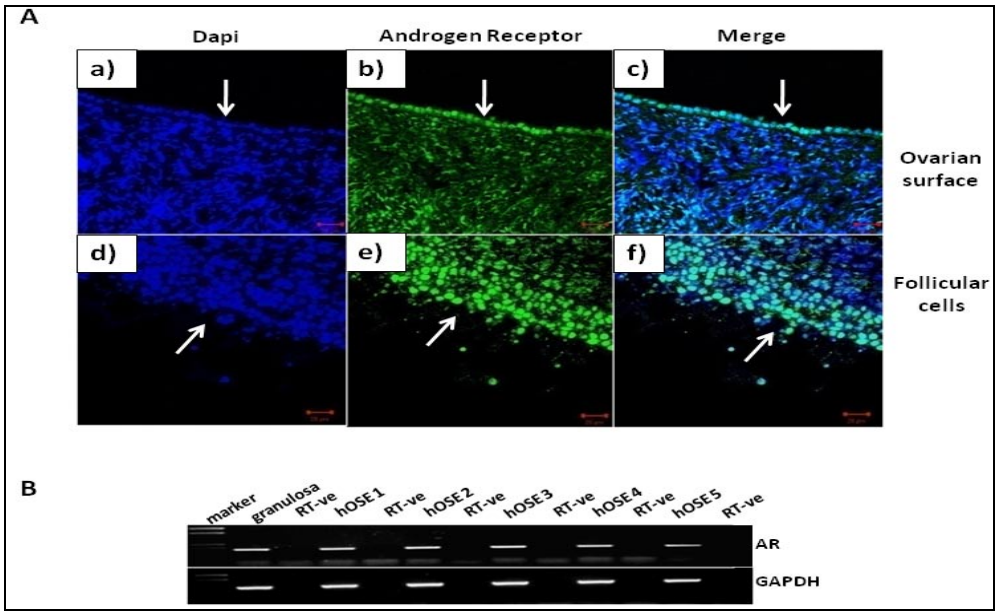


Figure 6.1: Expression of AR protein and mRNA in hOSE. A) Immunofluorescence to whole ovarian tissue for AR (green). White arrows in a-c denote hOSE cells, whereas arrows in d-f denote follicular cells. Nuclei were stained with Dapi (blue). Scale bars 20μm. B) sqPCR for AR mRNA (upper gel) and GAPDH mRNA (lower gel) in lutein-granulosa cells and five hOSE samples.

6.3.2 Expression of progesterone receptor in the human ovarian surface epithelium

Fluorescence immunohistochemistry was performed to immunodetect PR in whole ovarian tissue. PR (red) was immunodetected in the nuclei of the ovarian surface epithelial cells (Fig. 6.2A, b-c) as well as in the corpus luteum (Fig. 6.2A, e-f).

Moreover, sqPCR for PR mRNA expression was performed in five separate primary cultured hOSE cells. PR was expressed in five hOSE samples from five patients (Fig. 6.2B, upper gel). Lutein-granulosa cells were again used as a positive control (Fig. 6.2B, upper gel). cDNA integrity/quality was assessed with GAPDH mRNA (Fig. 6.2B, lower gel).

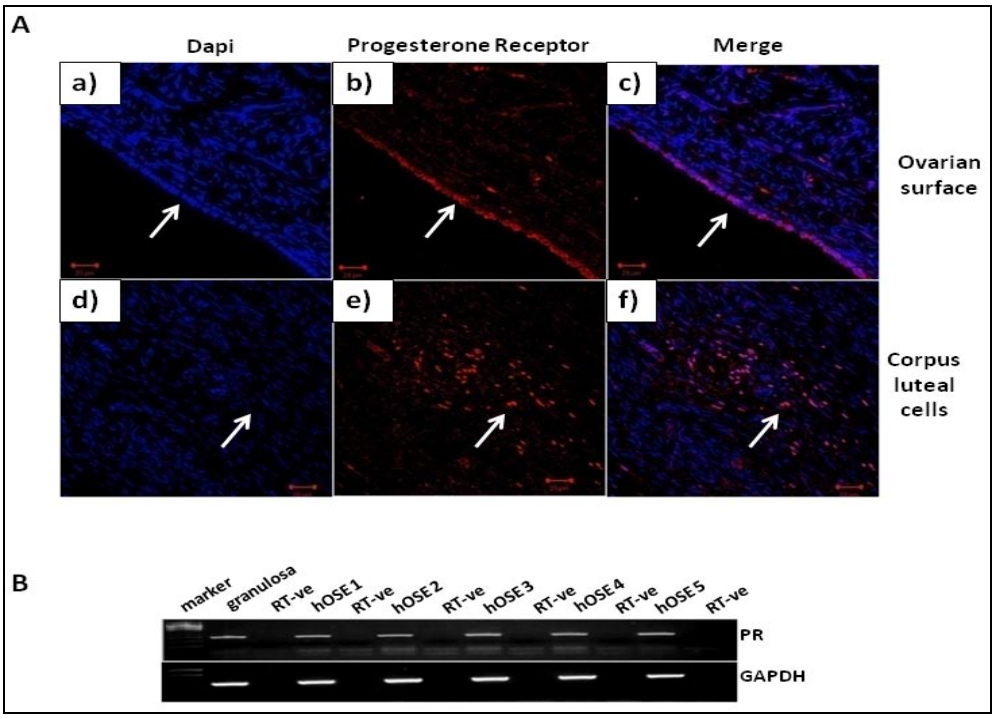


Figure 6.2: Expression of PR protein and mRNA in hOSE. A) Fluorescence immunohistochemistry in whole ovarian tissue shows nuclear immunodetection of PR (red) in hOSE and corpus luteum (arrows). Nuclei were stained with Dapi (blue). Scale bars represent 20μm. B) sqPCR was performed in lutein-granulosa cells and five hOSE samples to investigate mRNA expression of PR (upper gel) and GAPDH (lower gel).

6.3.3 Effects of IL-1α on AR and PR mRNA expression in primary hOSE cells

Taqman qPCR was performed in primary hOSE cell cultures to investigate the effects of IL-1α (0.5ng/mL) on AR mRNA and PR mRNA. Combined data from four independent hOSE samples that were treated with IL-1α for 0, 12, 24 and 48h showed that IL-1α did not affect basal mRNA levels of AR (Fig. 6.3A) or PR (Fig. 6.3B).

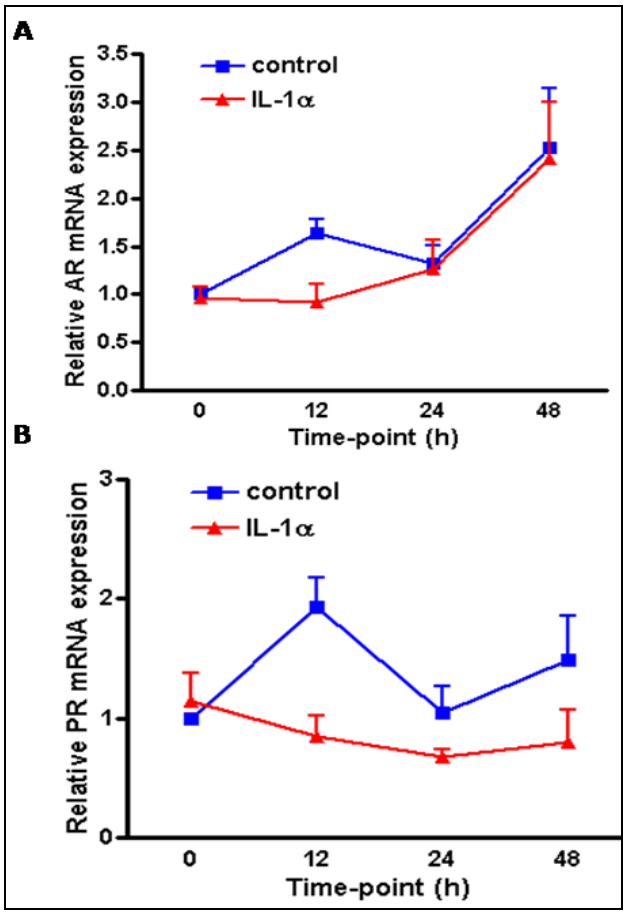


Figure 6.3: Effect of IL-1α on AR and PR mRNA expression. Combined data of 4 independent experiments. Time-course studies to investigate the effects of IL-1α (0.5ng/mL) on AR mRNA (A) and PR mRNA (B) (n=4).

6.3.4 Effects of IL-4 on AR and PR mRNA expression in primary hOSE cells

To further investigate androgen and progesterone action in hOSE cells, we treated cell monolayers with IL-4 (0.5ng/mL) for 0, 12, 24 and 48h and assessed its effects on AR and PR mRNA levels. IL-4 treatment did not affect PR mRNA (Fig. 6.4B), but it down-regulated AR mRNA (Fig. 6.4A). AR mRNA suppression appeared slow and progressive with a significant decrease observed after 48h treatment with IL-4 (2-fold inhibition; Fig. 6.4B, n=3, b=$p<0.05$).

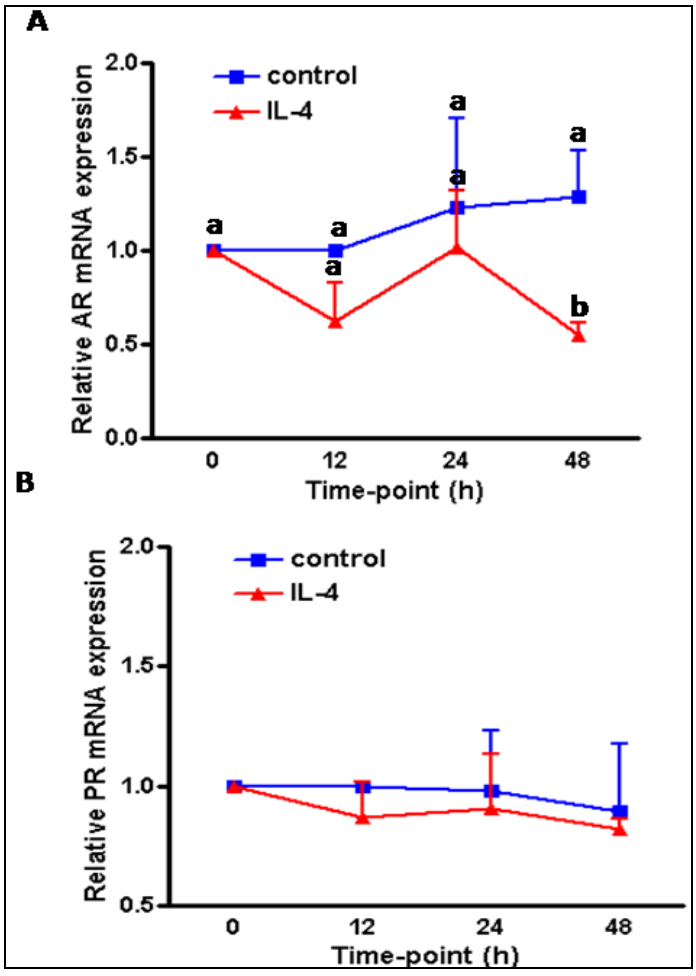


Figure 6.4: Effect of IL-4 on AR and PR mRNA expression. Combined data of 3 independent replicates. Primary hOSE cells were treated with IL-4 (0.5ng/mL) for 0, 12, 24 and 48h. Then, effects on AR (A) and PR (B) mRNA were measured with Taqman qPCR (n=3, b=$p<0.05$).

6.3.5 The effects of IL-1α and IL-4 co-treatment on AR and PR mRNA expression in primary hOSE cells

To further confirm the responses of IL-1α and IL-4 to AR and PR mRNA levels, we treated the same hOSE sample with both cytokines (0.5ng/mL) for 48h in five independent replicates. Treatment with IL-1α did not affect either AR mRNA (Fig. 6.5A) or PR mRNA (Fig. 6.5B), whereas IL-4 suppressed AR mRNA with no influence on PR mRNA (Fig. 6.5A and 6.5B, respectively). Co-treatments did not affect the inhibitory IL-4 effect on AR mRNA (Fig. 6.5A, n=5, b=$p<0.05$).

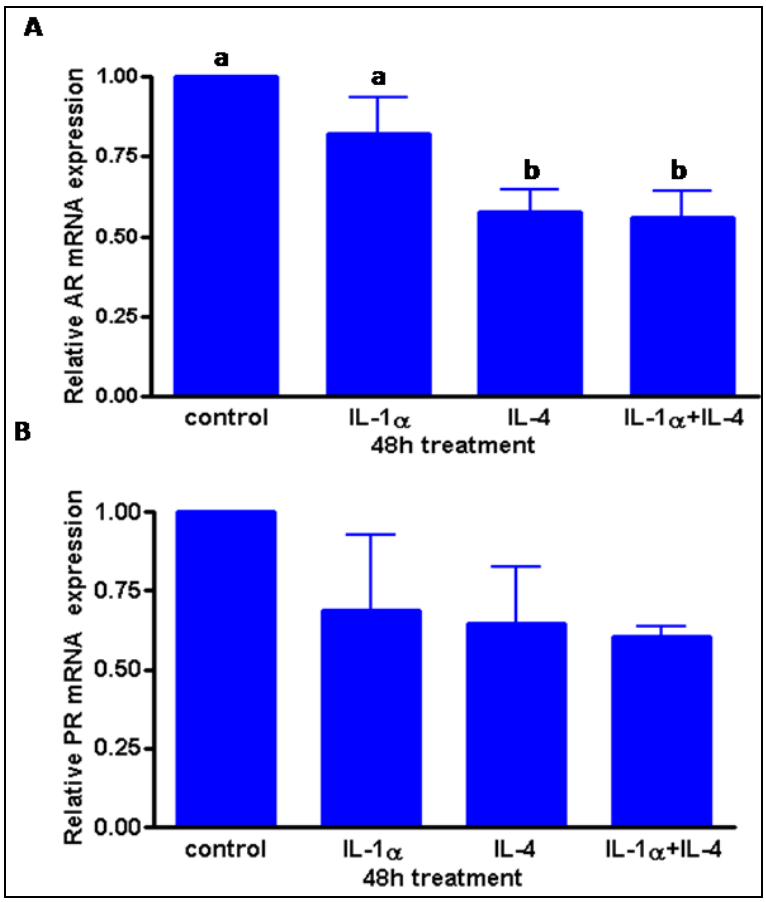


Figure 6.5: The effects of IL-1α and IL-4 on AR and PR mRNA expression. Combined data from 5 independent hOSE samples. The same hOSE sample was treated with both IL-1α and IL-4 and the effects on AR mRNA and PR mRNA levels were assessed with Taqman qPCR (n=5, b=$p<0.05$).

6.3.6 The dose-dependent effects of IL-4 on AR mRNA and AR protein in primary hOSE cells

To further investigate the effect of IL-4 on AR mRNA expression, we treated primary hOSE cells with increasing doses of IL-4 (0.02, 0.1, 0.5ng/mL) for 48h (Fig. 6.6A). Taqman qPCR showed that the suppression of AR mRNA levels relative to untreated control was observed only at the highest dose of IL-4 treatment (0.5ng/mL) (Fig. 6.6A, n=4, b=$p<0.05$). To assess if this effect also remained at the protein level, we treated hOSE cells with 0.5ng/mL and 5ng/mL IL-4 for 48h and we assessed the effects of IL-4 on AR protein extracts using western immunoblotting (Fig. 6.6B). Moderate inhibition of AR protein was demonstrated only when 5ng/mL of IL-4 was added (Fig. 6.6B, upper blot). Protein loading was ascertained with probing for actin (Fig. 6.6B, lower blot). The specificity of the AR antibody was assessed with T47D cells as described in Chapter 2.

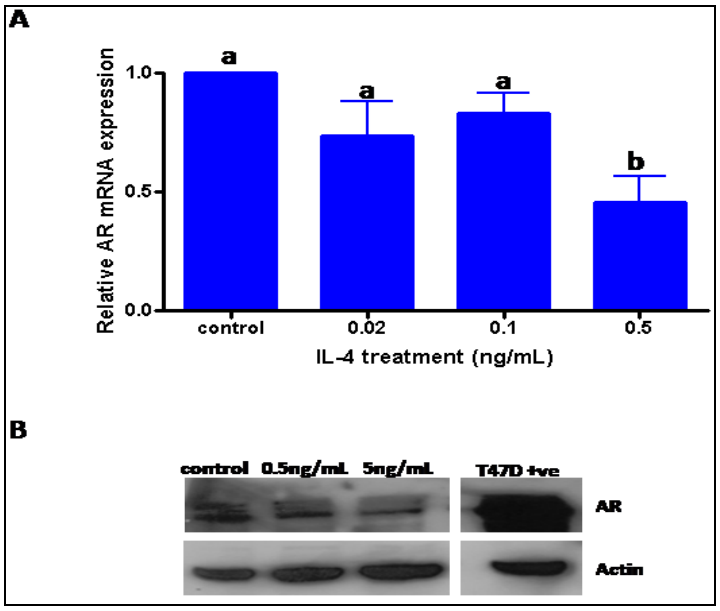


Figure 6.6: The effects of IL-4 on AR mRNA and protein in hOSE cells. A) Combined data of 4 hOSE cell cultures. Cells were treated with increasing doses of IL-4 in a dose-response manner (0.02-0.5ng/mL) for 48h and effects on AR mRNA were measured. B) A representative blot shows effects of IL-4 (0.5 and 5ng/mL) on AR protein. Actin was used as a loading control. Human T47D cell line was used as a positive control for AR protein.

6.3.7 Signalling pathways involved in the transduction of IL-4-mediated AR mRNA expression in primary hOSE cells

As discussed in Chapter 1, IL-1α and IL-4 mediate their effects through binding to their cognate cell surface receptors IL-1R1 and IL-4R, respectively, followed by transactivation of the latter and initiation of signalling cascades. To investigate the signalling pathways involved in IL-4 suppression of AR mRNA, we treated primary hOSE cells with IL-4 in the presence or absence of inhibitors specific for ERK1/2, p38 MAPK, NF-κB, PI-3K and STAT-6 signalling pathways.

6.3.7.1 The role of the MAPK pathways in IL-4 suppression of AR mRNA levels

In order to investigate if IL-4-decreased AR mRNA expression involved the ERK1/2 pathway, we treated hOSE cells with IL-4 (0.5ng/mL) in the presence or absence of PD98059 (50μM) for 48h. Taqman qPCR for AR mRNA, showed that inhibition of the ERK1/2 pathway did not reverse suppression of AR mRNA levels by IL-4 treatment (Fig. 6.7A, n=3, b=$p<0.05$).

Investigation of a potential role of the p38 MAPK pathway in IL-4-mediated AR mRNA was achieved through inhibition of the p38 MAPK pathway with addition of SB203580 (10μM) in the presence of IL-4 (0.5ng/mL) for 48h. Taqman qPCR in 4 independent experiments demonstrated that SB203580 reversed the IL-4 suppression of AR mRNA levels, whilst it did not affect AR mRNA levels in the absence of IL-4 (Fig. 6.7B, n=4, b=$p<0.05$).

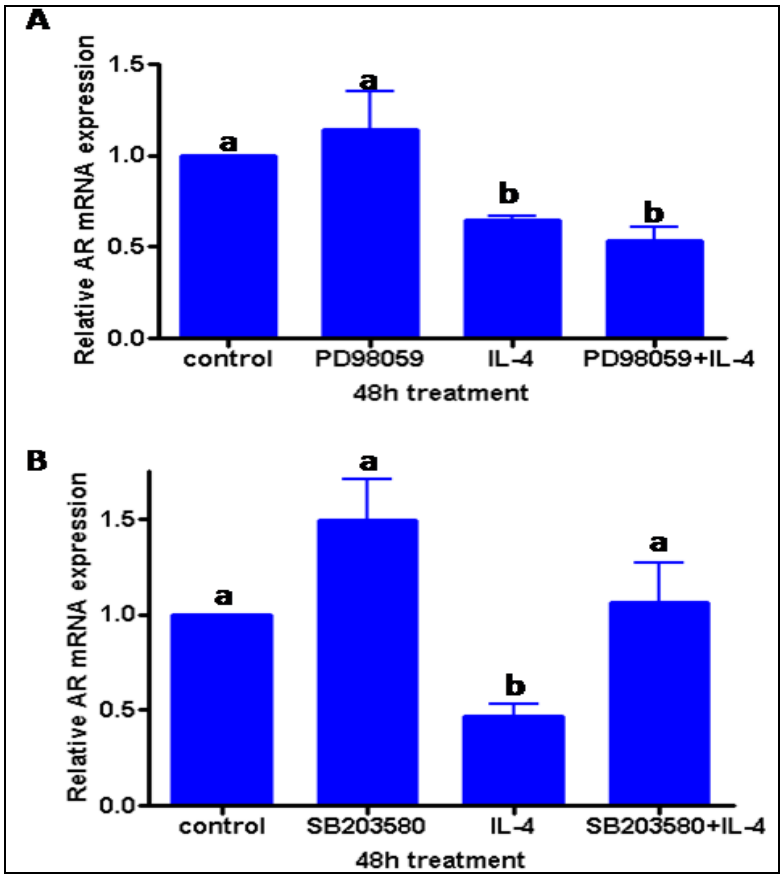


Figure 6.7: The effects of (A) an ERK1/2 and (B) a p38 MAPK pathway inhibitors on AR mRNA levels. Combined data of 3 (A) and 4 (B) independent experiments. Effects of inhibitors of ERK1/2, PD98059 (50μM) (A) and p38 MAPK, SB203580 (10μM) (B) pathways on the AR mRNA levels in the presence or absence of IL-4 (0.5ng/mL) for 48h (b=$p<0.05$).

6.3.7.2 The role of the NF-κB pathway in IL-4 suppression of AR mRNA levels

Primary hOSE cells were treated with IL-4 (0.5ng/mL) in the presence or absence of BAY117082 (1μM) for 48h to determine if an NF-κB inflammatory pathway plays a role in the suppression of AR mRNA levels by IL-4. Although IL-4 in the presence of the inhibitor did not significantly inhibit AR mRNA relative to untreated control cells, cells treated with both IL-4 plus inhibitor were not significantly different from the IL-4-treated cells (Fig. 6.8; n=4, b=$p<0.05$).

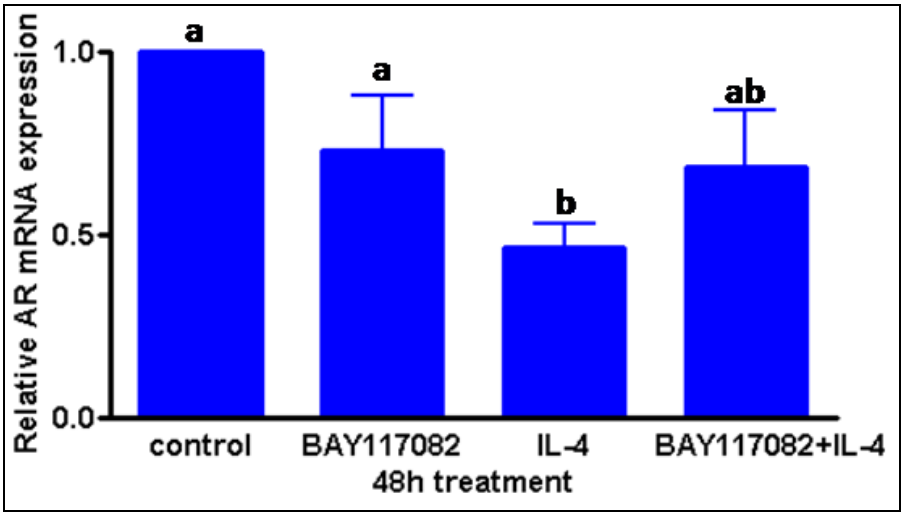


Figure 6.8: The effect of an NFκ-B pathway inhibitor on AR mRNA levels. Combined data of 4 independent replicates. Taqman qPCR was performed to investigate whether the inhibitor of the NF-κB pathway, BAY117082 (1μM) affects IL-4 (0.5ng/mL; 48h) suppression of AR mRNA levels (n=4, b=$p<0.05$).

6.3.7.3 The role of the PI-3K pathway in IL-4 suppression of AR mRNA levels

IL-4 treatment (0.5ng/mL) for 48h was applied to primary hOSE samples in the absence or presence of LY294002 (10μM) in order to investigate any potential role of the PI-3K pathway in IL-4-decreased AR mRNA expression. As established with Taqman qPCR, abrogation of AR mRNA levels by IL-4 were not affected by inhibition of the PI-3K pathway (Fig. 6.9; n=3, b=$p<0.05$).

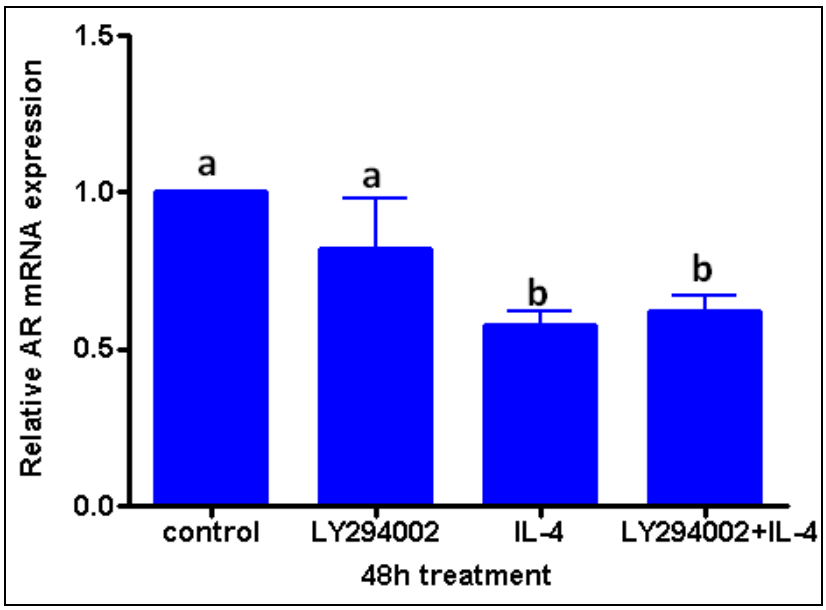


Figure 6.9: The effect of a PI-3K pathway inhibitor on AR mRNA levels. Combined data of 3 independent experiments. Taqman qPCR was performed to investigate whether the inhibitor of the PI-3K pathway, LY294002 (10μM) affects IL-4 suppression of AR mRNA levels (n=3, b=$p<0.05$).

6.3.7.4 The role of the STAT-6 pathway in IL-4 suppression of AR mRNA levels

IL-4 treatment (0.5ng/mL) for 48h was applied to primary hOSE samples in the absence or presence of leflunomide (100μM) in order to investigate any potential role of the STAT-6 pathway in IL-4 abrogation of AR mRNA levels. As determined by Taqman qPCR, IL-4 action on AR mRNA levels was not reversed by inhibition of the STAT-6 pathway (Fig. 6.10; n=3, b=$p<0.05$).

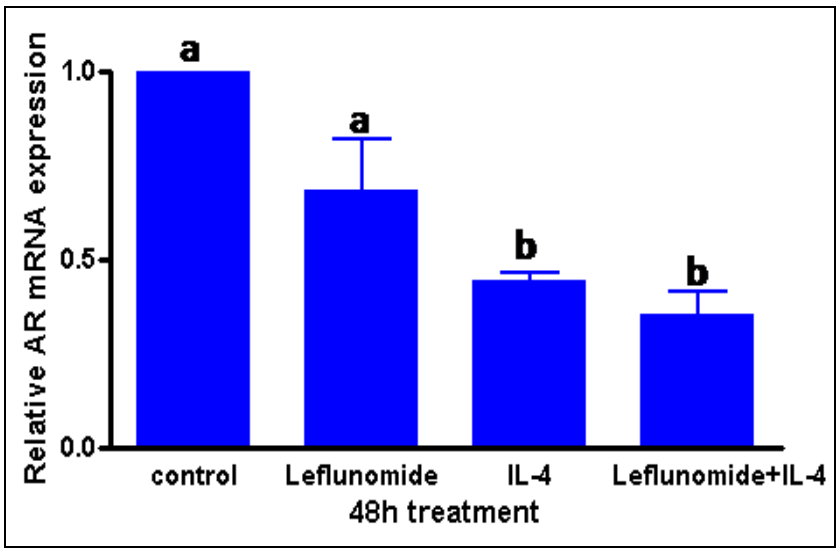


Figure 6.10: The effect of a STAT-6 pathway inhibitor on AR mRNA levels. Combined data of 3 independent experiments with distinct primary hOSE cells. Taqman qPCR was performed to investigate whether the inhibitor of the STAT-6 pathway, leflunomide (100μM) affects IL-4-suppressed AR mRNA levels (n=3, b=$p<0.05$).

6.3.8 Summary of the effects of IL-1α and IL-4 on AR and PR mRNA and protein expression

Table 6.4 IL-1α and IL-4 effects on AR mRNA/protein and PR mRNA expression

Treatment	AR mRNA/protein	PR mRNA
IL-1α	n/s	n/s
IL-4	↓	n/s
IL-1α+IL-4	↓	n/s

n/s: not significant, ↓: down-regulation

Table 6.5 Effects of signalling pathway inhibitors on transcriptional regulation of target genes

Treatment	Target pathway	AR mRNA
PD98059	ERK1/2	n/s
SB203580	p38 MAPK	n/s
BAY117082	NF-κB	n/s
LY294002	PI-3K	n/s
Leflunomide	STAT-6	n/s
IL-4		↓
PD98059+IL-4	ERK1/2	↓
SB203580+IL-4	p38 MAPK	n/s
BAY117082+IL-4	NF-κB	n/s
BAY117082+IL-4 vs IL-4	NF-κB	n/s
LY294002+IL-4	PI-3K	↓
Leflunomide+IL-4	STAT-6	↓

↓: down-regulation, n/s: not significant

6.4 Discussion

In previous Chapters we demonstrated 3β-HSD expression in the ovarian surface, suggesting that this ovarian compartment is capable of producing PR- and/or AR-activating ligands. Herein, we illustrate that besides 3β-HSD, AR and PR are also expressed in hOSE cells. More importantly, we have demonstrated that IL-4 suppressed AR mRNA and protein levels without affecting expression of PR levels, suggesting that IL-4 sustains progesterone rather than androgen signalling in hOSE. Notably, this effect appeared to be mediated by an IL-4-induced p38 MAPK signalling pathway.

Fluorescence immunohistochemistry confirmed the presence of AR and PR immunoreactive proteins in the human ovarian surface epithelium, confirming previous reports (Edmondson *et al.* 2002, Li *et al.* 2003). These data show that 3β-HSD active ligands can act through binding to the cognate AR and PR receptors. As such, the human ovarian cell surface possesses the enzyme and receptor machinery for functional signalling of the 3β-HSD potent PR ligand, progesterone and the weak AR ligand, androstenedione. The latter can subsequently be converted to the more potent AR ligand, testosterone, a reaction catalysed by 17β-HSD that is also expressed in hOSE (Rae *et al.* 2004b). After establishment of AR and PR localisation in the ovarian cell surface, it was next demonstrated that measurable AR and PR mRNA levels remain in primary cultures of hOSE cells, allowing therefore a study of the regulation of these receptors *in vitro* using this cell culture system.

Although previous studies have established the responsiveness of hOSE to androgen and progesterone, it has not been determined whether AR and PR levels are affected during post-ovulatory injury and repair of hOSE cells. To this end, we treated hOSE cell monolayers with IL-1α to mimic post-ovulatory wounding of hOSE or with IL-4 to mimic post-ovulatory repair of hOSE and then assessed AR and PR mRNA expression levels relative to untreated control cells. IL-1α did not affect basal mRNA levels of either AR or PR, supporting the hypothesis that during

post-ovulatory injury of hOSE there is not only a balance in local steroid biosynthesis but also in access of ligands to the cognate nuclear receptors. As such, at the apex of ovulation, when IL-1α is produced, steroid biosynthesis and downstream signalling does not alter and this might well be part of the mechanisms that monitor homeostasis of the ovarian cell surface during injury.

We also found that IL-4 significantly suppressed AR mRNA and protein levels, albeit at a progressive and rather sluggish manner as revealed in time-course and dose-response studies. On the other hand, IL-4 did not alter PR mRNA levels. Collectively, these effects imply that IL-4 sustains progesterone rather than androgen action in hOSE during the post-ovulatory healing processes. The importance of this finding is subject to the general concept proposed in Chapter 4 that IL-4 plays a role in post-ovulatory repair by monitoring proliferation of integral only hOSE cells, whilst it commits genetically damaged cells to die. It appears that IL-4 fulfils this effect in two stages; firstly, it stimulates local 3β-HSD activity, thereby triggering formation of progestogens and androgens and secondly, it sustains progesterone action through suppression of AR function. Hence, during the luteal phase of the menstrual cycle when repair takes place, IL-4 activity on hOSE allows progesterone to induce cell apoptosis and repair of genetically scarred hOSE cells (Murdoch 1998, Murdoch & Van Kirk 2002). Notably, a role of IL-4 in mediation of stress inflammatory responses has been also demonstrated in bovine adrenal cells, where IL-4 increased ACTH-induced release of the anti-inflammatory cortisol through abolishment of ACTH-stimulated secretion of androgens (Woods & Judd 2008). Also, another study concluded that the expression of IL-4 and AR was negatively correlated in fibroblast cells collected from patients with gingival overgrowth or control patients (Huang *et al.* 2003). In general, it is possible that a way by which IL-4 alleviates inflammatory responses could be through the suppression of AR transactivation. This could have profound implications in the aetiology of EOC where a positive link between androgens and development of the disease has been constantly proposed (Edmondson *et al.* 2002, Evangelou *et al.* 2000, Evangelou *et al.* 2003, Risch 1998, Shaw *et al.* 2001). However, it should be noted at this point, that

in the prostate cancer cell line LNCaP, IL-4 has been shown to activate rather than suppress AR activity (Lee *et al.* 2003), showing that effects of IL-4 on AR might differ between cell types or importantly might alter between normal and malignant tissues. This could be a mechanism of tumour cells to escape from T cell surveillance, an aspect that was discussed in Chapter 1 (Section 1.3).

Specific inhibitors for several signal transducing pathways were used to identify the mechanisms through which IL-4 suppresses AR function, at least regarding mRNA transcriptional levels. These studies showed that only the p38 MAPK signalling pathway appears essential for the transcriptional regulation of AR mRNA by IL-4. Surprisingly, the STAT-6 and PI-3K signalling pathways that are commonly activated by IL-4 did not appear to be involved in IL-4-attenuated AR mRNA expression. It is noteworthy, therefore, that IL-4 mediates its effects on several responsive genes by the activation of different panels of signalling pathways. Despite the fact that IL-4 can influence the regulation of AR not only in hOSE cells, but also in other cell systems (see above), there is no evidence of STAT-6 involvement in these effects and there are also no reports of STAT-6 recognition sites in the promoter of AR gene. Moreover, inhibition of NF-κB pathways partially but not significantly reversed IL-4-decreased AR mRNA expression levels. Based on the effects of this pathway on the IL-4-induced 3β-HSD mRNA species, it is unlikely that NF-κB is involved. However, the nature of this borderline effect does not allow us to draw any firm conclusion from the present data.

The potential involvement of the p38 MAPK pathway in IL-4 suppression of AR mRNA levels further confirmed the anti-inflammatory nature that this pathway displays when induced by IL-4R transactivation. As discussed in Chapter 5, the p38 MAPK pathway appeared to mediate anti-inflammatory responses in hOSE cells such as IL-4-elevated 3β-HSD2 mRNA expression levels. Moreover, it was demonstrated to be part of IL-1α-related anti-inflammatory responses as revealed by IL-1α-induced 3β-HSD2 (Chapter 5) and 11β-HSD1 (Rae M.T., unpublished observations) mRNA expression levels. This may well reflect a negative feedback

loop mechanism that hOSE cells use to alleviate tissue damage during ovulation-associated wounding.

In conclusion, we have demonstrated that AR and PR mRNA levels are not affected during post-ovulatory wounding of hOSE cells, suggesting a balance in access of 3β-HSD-associated ligands to AR and PR. Also, IL-4 attenuated AR mRNA and protein levels without affecting PR mRNA expression, thereby favouring progesterone rather than androgen signalling in post-ovulatory repair. Importantly, this effect required an activated p38 MAPK pathway, further demonstrating the anti-inflammatory nature of this pathway in hOSE cells. An illustration of the proposed model is depicted in Fig. 6.11.

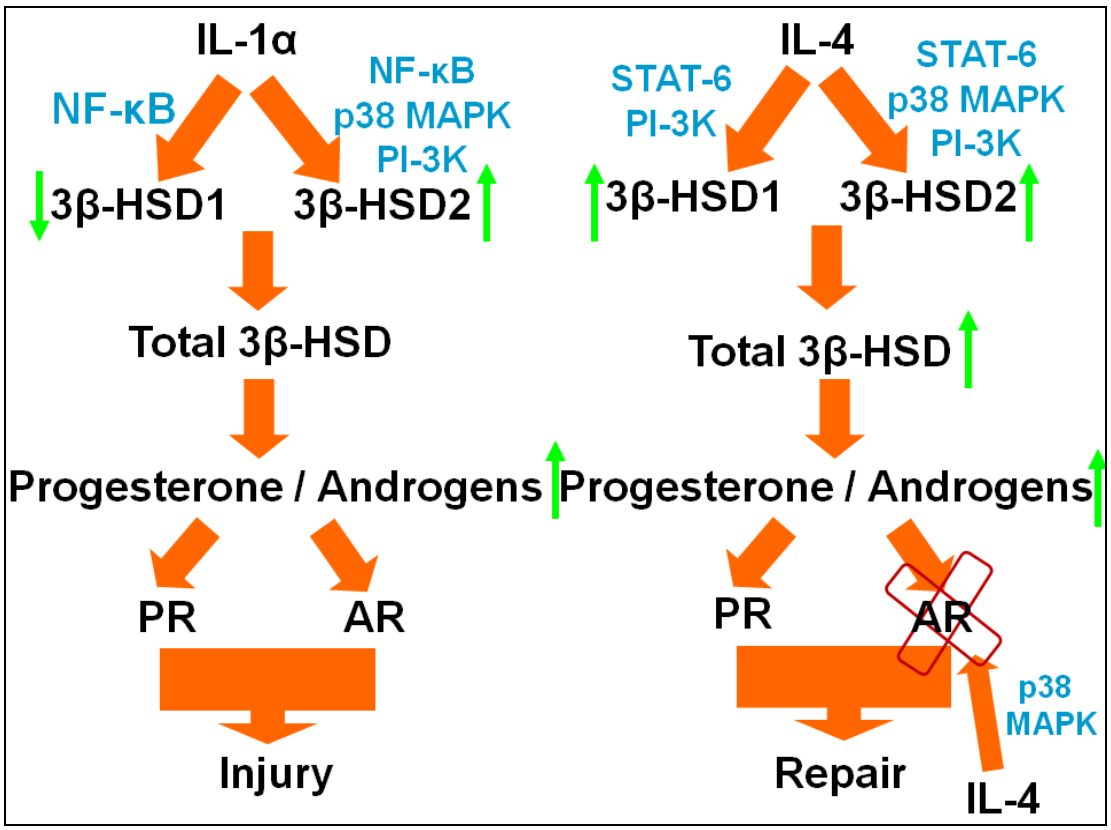


Figure 6.11: Regulation of steroid signalling by IL-1α and IL-4 in the human ovarian surface epithelium. Schematic illustration of the proposed model and mechanisms regarding the role of IL-1α and IL-4 in steroid biosynthesis and downstream signalling through the cognate receptors. Absence of arrow denotes no significance.

Chapter 7

Regulation of cyclooxygenase 2 and lysyl oxidase by cytokines. Further support for an anti-inflammatory role of IL-4 in the human ovarian surface epithelium

7.1 Introduction

In the previous Chapters it has been shown that IL-1α and IL-4 differentially regulate the expression of steroid-related genes such as 3β-HSD1, 3β-HSD2 and AR during post-ovulatory injury and repair of the ovarian surface. It is widely accepted that IL-1α exerts pro-inflammatory effects to prepare the ovarian cell surface to host ovulation-associated wounding. As such, at the apex of ovulation, the pro-inflammatory-related genes, COX-2 and MMP-9 are up-regulated (Murdoch 2000, Rae *et al.* 2004a, Rask *et al.* 2006), whilst 3β-HSD1 and peroxisome proliferator-activated receptor-γ (PPAR-γ), both exerting anti-inflammatory actions, are down-regulated (Rae *et al.* 2004b). Simultaneously, genes involved in counteracting tissue damage such as 11β-HSD1 mRNA (Yong *et al.* 2002) and 3β-HSD2 mRNA (see Chapter 4) also appear to be induced in response to IL-1α to trigger the intracrine generation of anti-inflammatory glucocorticoid and progesterone, respectively. In particular, cortisol has been demonstrated to contribute to the alleviation of post-ovulatory damage of hOSE as it triggered elevation of the IL-1α-induced 11β-HSD1 mRNA and blocked IL-1α-induced COX-2 mRNA (Rae *et al.* 2004a) and IL-1α-induced MMP-9 mRNA (Rae *et al.* 2008). Moreover, progesterone has been established to suppress IL-1α-increased COX-2 mRNA levels, attesting its anti-inflammatory nature in hOSE cells (Rae *et al.* 2004a). Additionally, progesterone has been illustrated to play a role in apoptosis and repair of the ovarian cell surface (Murdoch 1998, Murdoch & Van Kirk 2002, Murdoch *et al.* 2001), indicative of its essential action during post-ovulatory remodelling of hOSE, so that only genetically integral cells proliferate to restore the wounded tissue. As shown in Chapter 4, intracrine generation of progesterone is driven by the 3β-HSD activity that is elevated substantially by IL-4 treatment of hOSE cells, suggesting that this cytokine may exert anti-inflammatory effects during the post-ovulatory healing of hOSE.

Also, important in the successful regeneration of hOSE following ovulation-associated wounding and tissue remodelling, is the prevention of uncontrolled cell proliferation that in turn could lead to cell adhesion and invasion, functions that

might well contribute to tumourigenesis and metastasis. In that context, successful deposition of the extracellular matrix (ECM) during post-ovulatory repair could be an efficient mechanism for hOSE to restore homeostasis. Given the anti-inflammatory nature of IL-4, one might speculate that IL-4 could also contribute to ECM deposition as part of its role in the post-ovulatory healing processes at the ovarian cell surface. An ECM-related gene that has been shown to be altered during hOSE-associated post-ovulatory wounding processes is lysyl oxidase (LOX) (Rae *et al.* 2004b).

LOX is an extracellular copper-containing enzyme that catalyses the oxidative deamination of tropoelastin and procollagen for the subsequent cross-linkages of elastin and collagen fibrils (Bedell-Hogan *et al.* 1993). This process is fundamental for the biosynthesis of a functional ECM. LOX has been reported to be secreted as a N-glycosylated 50kDa precursor that is post-translationally cleaved by procollagen C-terminal proteinase (PCP) activity to a non-glycosylated 35kDa active enzyme (Trackman *et al.* 1992, Uzel *et al.* 2001). Of the two procollagen proteinases - the mammalian Toloid (mTLD) and its shorter variant bone morphogenetic protein-1 (BMP-1) - encoded by the *Bmp1* gene - BMP-1 has been reported to process pro-lysyl oxidase more efficiently (Hartigan *et al.* 2003, Uzel *et al.* 2001). Interestingly, PCP/BMP-1 activity is enhanced by its interaction with an extracellular multidomain glycoprotein, the PCP enhancer (PCPE) (Ricard-Blum *et al.* 2002).

LOX has been shown to be secreted in multiple cell types including gingival fibroblasts (Hong *et al.* 1999), osteosarcoma cells (Uzel *et al.* 2000) and aortic smooth muscle cells (Maki *et al.* 2002). Notably, impaired LOX function is correlated with colorectal cancer (Csiszar *et al*., 2002). Also, LOX has been shown to inhibit ERK1/2 and Akt mitogenic activities in human lung cancer cells as well as in a mouse fibroblast cell line, whilst it was shown to block NF-κB protein and the anti-apoptotic bcl-2 gene in the PANC-1 pancreatic cancer cell line (Jeay *et al.* 2003, Wu *et al.* 2007). It has been also documented that a mechanism through which tumourigenesis can develop is by methylation-induced silencing of LOX. This

appears to be the case in several cancer cell lines such as colon, lung, ovarian, gastric and pancreatic cancer cell lines, establishing LOX as a tumour suppressor gene (Kaneda *et al.* 2004).

Therefore, in order to further support the importance of IL-4 function in post-ovulatory tissue remodelling of hOSE, we investigated its potential effects on alleviation of inflammation and on ECM deposition. To achieve this, we selected two key genes as paradigms for these two responses; first, we assessed the effects of IL-4 on the pro-inflammatory COX-2 mRNA in the presence or absence of IL-1α and secondly, we asked whether LOX mRNA expression levels are altered by IL-4 differently in the presence or absence of IL-1α.

7.2 Subjects and Methods

To further elaborate the anti-inflammatory nature of IL-4 in hOSE, we assessed the effects of this cytokine on the pro-inflammatory COX-2 mRNA and the ECM-related LOX mRNA expression levels in the presence or absence of IL-1α as a pro-inflammatory proxy. Primary hOSE cells were treated with IL-1α and IL-4 (0.5ng/mL) for 48h followed by cell harvest, RNA purification and quality/quantity assessment. Then, samples were submitted for Taqman qPCR of the target genes to compare treated with untreated hOSE samples.

To elucidate further the mechanisms through which these effects are exerted, we treated primary hOSE cell monolayers with IL-1α and IL-4 in the presence or absence of various transducing pathway inhibitors as described in Chapter 5. Afterwards, reverse transcription and Taqman qPCR were performed on RNA extracts to identify the signalling pathways that potentially may be involved in the mediation of LOX mRNA alterations in response to IL-1α and IL-4.

The basic clinical information of the patients who consented to provide tissue for our studies is presented in Tables 7.1 and 7.2.

Table 7.1 Clinical profile of patients whose hOSE were used for treatments with IL-1α and IL-4

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
38	7314	04/S1103/36	43	Oopherectomy	HMB	n/s	IL-1α+IL-4
8	5499	04/S1103/36	47	TAHBSO	Fibroids	n/s	IL-1α+IL-4
39	7326	04/S1103/36	43	TAH	Fibroids	n/s	IL-1α+IL-4
12	7383	04/S1103/36	43	TAHBSO	Fibroids	n/s	IL-1α+IL-4
37	7414	04/S1103/36	22	DiagLapar	Pelvic pain	n/s	IL-1α+IL-4

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectom, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days

Table 7.2 Clinical profile of patients whose hOSE were used to study pathway inhibitor effects

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
41	7320	04/S1103/36	47	TAHBSO	HMB	n/s	SB20
38	7332	1998/6/33	42	TAHBSO	Prophylactic	(21) Luteal	SB20
23	7324	04/S1103/36	44	DiagLapar	Pelvic pain	(15) Follicular	SB20
39	7336	04/S1103/36	40	TAH	Prophylactic	(22) Luteal	SB20
44	9018	04/S1103/36	33	DiagLapar	Mid-cycle pain	(19) Luteal	LY29
45	5517	04/S1103/36	43	LapSter	Fibroids	(21) Luteal	LY29
10	5536	04/S1103/36	23	DiagLapar	Dysmenorrhoea	(4) Follicular	LY29

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, LapSter: laparoscopic sterilisation, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days. SB20: SB203580, LY29: LY294002

7.3 Results

7.3.1 The effects of IL-1α and IL-4 on pro-inflammatory COX-2 mRNA expression in primary hOSE cells

Primary hOSE cells that were treated with IL-1α, IL-4 or a combination of both (0.5ng/mL) for 48h were harvested and assessed for mRNA expression levels of COX-2. Untreated cells were used as controls. As expected from previous reports, IL-1α significantly augmented COX-2 mRNA levels with an average 7-fold increase (Fig. 7.1; n=5, b=$p<0.001$). On the other hand, IL-4 treatment did not alter COX-2 mRNA levels. However, when IL-4 was applied in the presence of IL-1α, IL-1α-induced COX-2 mRNA was significantly suppressed (Fig. 7.1, b vs c $p<0.05$).

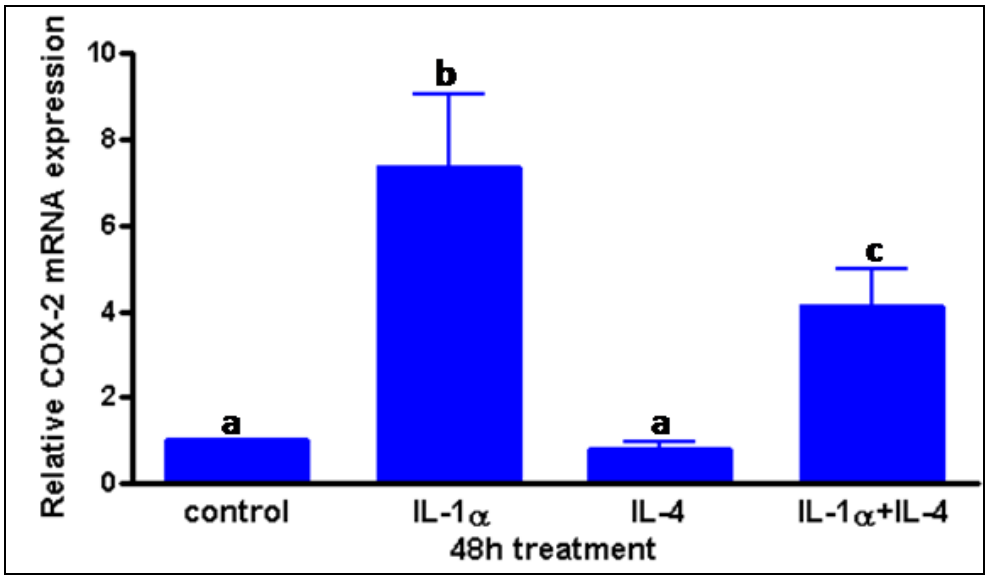


Figure 7.1: The effects of IL-1α and IL-4 on COX-2 mRNA expression. Combined data from 5 independent hOSE samples. Taqman qPCR was performed to assess COX-2 mRNA levels in response to IL-1α, IL-4 or both (0.5ng/mL) for 48h. (b=$p<0.001$, c=$p<0.05$, b vs c $p<0.05$).

7.3.2 The effects of IL-1α and IL-4 on LOX mRNA expression in primary hOSE cells

We then assessed alterations of LOX mRNA levels in RNA extracts from primary hOSE cells treated with IL-1α, IL-4 or both (0.5ng/mL) for 48h relative to untreated control samples. Taqman qPCR in five separate hOSE samples revealed that both IL-1α and IL-4 had a stimulatory effect on LOX mRNA levels relative to untreated control samples. IL-1α increased LOX mRNA levels at least 2 times, whereas IL-4 had a mean 6-fold stimulatory effect. Combination of both cytokines did not result in an additive effect (Fig. 7.2; n=5, b=$p<0.05$, c=$p<0.01$).

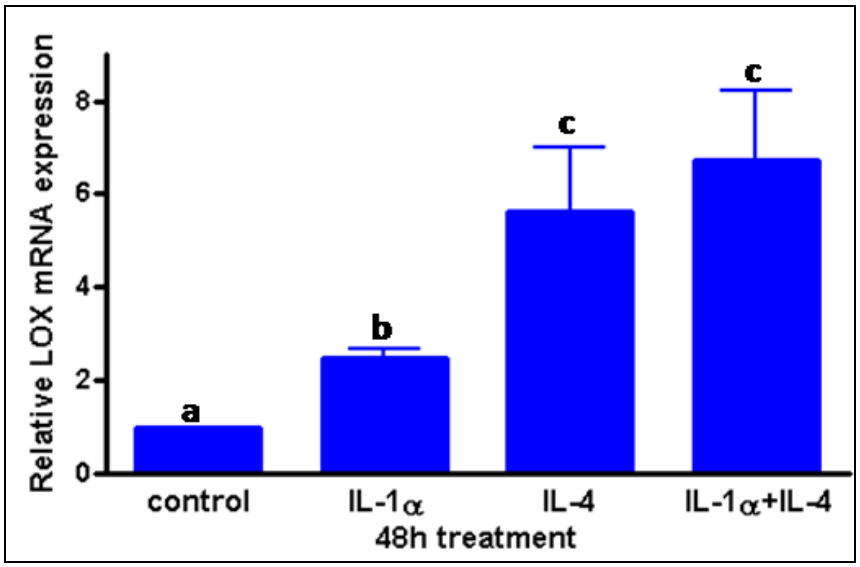


Figure 7.2: The effects of IL-1α and IL-4 on LOX mRNA expression. Combined data from 5 independent hOSE samples. Taqman qPCR was performed to assess LOX mRNA levels in response to IL-1α, IL-4 or both (0.5ng/mL) for 48h. (b=$p<0.05$, c=$p<0.01$).

7.3.3 Signalling pathways involved in the transduction of IL-1α and IL-4-mediated LOX mRNA expression in primary hOSE cells

As described in Chapter 5, in order to elucidate the mechanisms through which IL-1α and IL-4 exert their effects on the transcriptional regulation of responsive genes, primary hOSE cells were treated with cytokines in the presence or absence of specific pathway inhibitors. From the panel of the pathway inhibitors tested, namely ERK1/2, p38 MAPK inhibitors, NF-κB, PI-3K and STAT-6 pathway inhibitors, only p38 MAPK and PI-3K were identified as intermediate regulators of the IL-1α and/or IL-4-induced machinery for mediation of transcriptional regulation of LOX mRNA. As such, data for only these two pathways are demonstrated herein.

7.3.3.1 Regulation of IL-1α and IL-4-mediated LOX mRNA expression by the p38 MAPK signalling pathway

Primary hOSE cells were treated with IL-1α or IL-4 (0.5ng/mL) in the presence or absence of an inhibitor of the p38 MAPK pathway, SB203580 (10μM) for 48h. Taqman qPCR of mRNA from treated and untreated hOSE cell monolayers revealed that IL-1α-induced LOX mRNA was suppressed when SB203580 was included. Similarly, IL-4-induced LOX mRNA levels were suppressed approximately 2 times when SB203580 was present (Fig. 7.3; n=4, b=$p<0.05$, c=$p<0.01$).

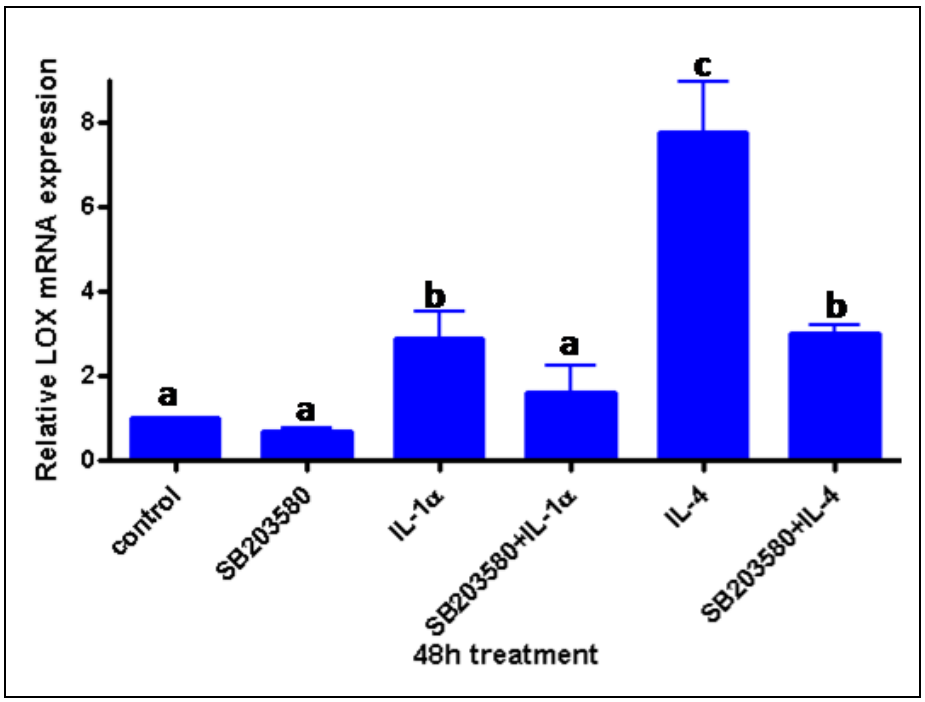


Figure 7.3: The effect of the p38 MAPK pathway inhibitor on LOX mRNA levels. Combined data of 4 independent hOSE samples. Taqman qPCR for LOX mRNA levels in cells treated with SB203580 (1μM) in the presence or absence of IL-1α or IL-4 (0.5ng/mL) for 48h (n=4, b=$p<0.05$, c=$p<0.01$).

7.3.3.2 Regulation of IL-1α and IL-4-mediated LOX mRNA expression by the PI-3K signalling pathway

Primary hOSE cell monolayers collected from 3 distinct patients were treated with LY294002 (10μM) in the presence of IL-1α or IL-4 (0.5ng/mL) for 48h. Assessment of LOX mRNA levels in target samples was achieved with Taqman qPCR. IL-4-induced LOX mRNA levels were suppressed when LY294002 was added. However, the inhibitor did not significantly affect IL-1α-induced LOX mRNA levels, albeit that LOX mRNA levels were slightly suppressed (Fig. 7.4; n=3, b, c=p<0.05, d=p<0.01).

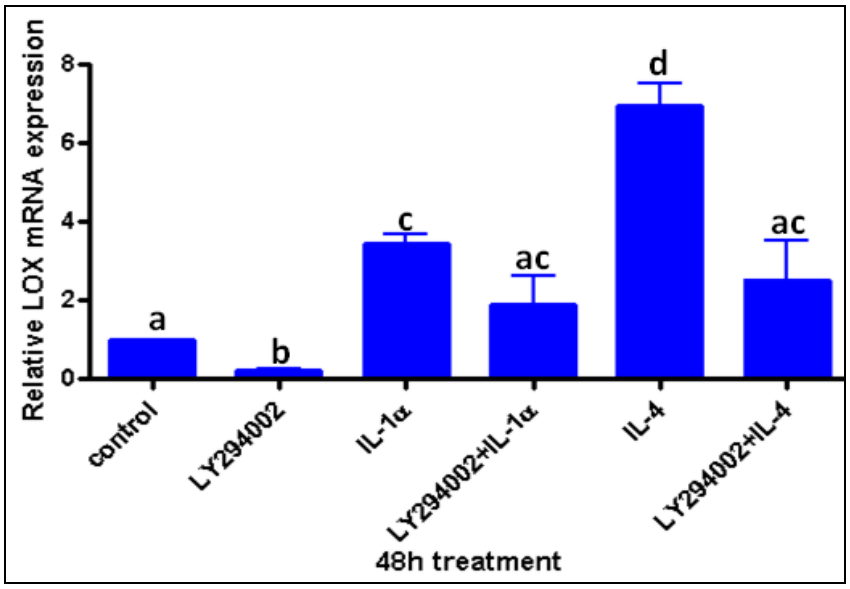


Figure 7.4: The effect of the PI-3K pathway inhibitor on LOX mRNA levels. Combined data of 3 independent hOSE samples. Taqman qPCR for LOX mRNA levels in cells treated with LY294002 (10μM) in the presence or absence of IL-1α or IL-4 (0.5ng/mL) for 48h (n=3, b, c=p<0.05, d=p<0.01).

7.3.4 Summary of effects of IL-1α and IL-4 on COX-2 and LOX mRNA expression

Table 7.3 Effects of cytokines and pathway inhibitors on COX-2 and LOX mRNA levels

Treatment	Target pathway	Target genes	
		COX-2 mRNA	LOX mRNA
IL-1α		↑	↑
IL-4		n/s	↑
IL-1α+IL-4		↑	↑
IL-1α+IL-4 vs IL-1α		↓	↑
SB203580	p38 MAPK	-	n/s
SB203580+IL-1α	p38 MAPK	-	n/s
SB203580+IL-4	p38 MAPK	-	↑
SB203580+IL-4 vs IL-4	p38 MAPK	-	↓
LY294002	PI-3K	-	↓
LY294002+IL-1α	PI-3K	-	n/s
LY294002+IL-1α vs IL-1α	PI-3K		n/s
LY294002+IL-4	PI-3K	-	n/s

n/s: not significant, -: not tested, ↑: up-regulation, ↓: down-regulation

7.4 Discussion

Herein, we provide further evidence of a role for IL-4 in post-ovulatory healing mechanisms in hOSE. IL-4 counteracts IL-1α induction of COX-2 mRNA. Also, it induces LOX mRNA, participating in ECM deposition, a critical process for integral regeneration of hOSE. The latter process appears to be mediated by p38 MAPK and PI-3K pathways. Importantly, IL-4-induced LOX expression is more profound than that occurring with IL-1α treatment, suggesting that expression of lysyl oxidase is essential during post-ovulatory repair of the stigma.

The treatment of primary hOSE cells with IL-4 alone did not affect COX-2 mRNA levels. However, in the presence of IL-1α, IL-4 attenuated IL-1α-induced COX-2 mRNA expression levels approximately 2-fold, suggesting that IL-4 inhibited prostaglandin synthesis during post-ovulatory repair, alleviating the degradation of connective tissue. At the same time, as shown in Chapter 4, IL-4 massively induced 3β-HSD activity and thus local progesterone biosynthesis. It is interesting that progesterone has been also documented to impede IL-1α-stimulation of COX-2 mRNA levels (Rae *et al.* 2004a). Therefore, it is notable that IL-4 directly impacts upon IL-1α-stimulated COX-2 mRNA levels, but at the same time it promotes intracrine generation of progesterone (through induction of 3β-HSD) that in turn also suppresses IL-1α-increased COX-2 mRNA expression (Rae *et al.* 2004a), indicative of a potential positive feedback loop mechanism.

The treatment of primary hOSE cells with IL-4 also resulted in the induction of LOX mRNA expression. Proper transcriptional and translational regulation of LOX is fundamental for the deposition of ECM and as such for the integral construction of the hOSE cell layer. Moreover, the LOX mRNA increase appeared to be triggered by IL-1α treatment as well, confirming previous studies (Rae *et al.* 2004b). Nevertheless, the effects of IL-4 were more profound than those of IL-1α. Importantly, combined effects of both cytokines were not additive, suggesting either independent action of the two proxies or a predominance of IL-4 action in LOX

transcriptional regulation. In physiology, LOX mRNA expression in response to IL-1α at the apex of ovulation is probably essential for minimisation of the injury of the inflammed tissue; however a profound elevation during post-ovulatory repair secures regeneration of the ovarian cell surface on a fully structured connective tissue. This is further supported by the finding that IL-4 abolished IL-1α-elevated COX-2 mRNA expression levels that potentially reflects an attenuated synthesis of prostaglandins that participate in degradation of the ECM. Additionally, we have shown that TGF-β1 up-regulated LOX mRNA in a benign immortalised hOSE cell line (Papacleovoulou *et al.,* unpublished observations), consistent with the anti-proliferative effects of TGF-β1 in hOSE cells (Choi *et al.* 2001c). Accordingly, LOX has been demonstrated to be elevated after ovulation in perch (Langenau *et al.* 1999) and rabbit ovary (Himeno 1986), indicating a potential role of LOX in post-ovulatory ovarian tissue remodelling. Moreover, in the rat ovary, LOX has been established to exert its effects on the maintenance of the ECM when the follicle was approaching final maturation (Harlow *et al.* 2003). Collectively, all these responses further support the potential function of LOX in the early stages of wound healing processes and protection from malignant transformation as shown in rat skin studies (Fushida-Takemura *et al.* 1996).

Importantly, p38 MAPK signalling appeared to mediate LOX mRNA transcriptional levels by IL-4 and IL-1α, once again attesting to the anti-inflammatory nature of this pathway at the ovarian cell surface. In essence, it is intriguing that NF-κB signalling pathways are not involved in IL-1α-stimulated LOX mRNA expression. This is consistent with previous studies proposing that the tumour suppressive effects of LOX are mediated through inhibition of the NF-κB pathway and associated pro-inflammatory responses (Jeay *et al.* 2003, Wu *et al.* 2007). Moreover, this is also consistent with our speculation that IL-4 antagonises IL-1α pro-inflammatory responses through inhibition of NF-κB and activation of p38 MAPK pathway (see Chapter 5). Surprisingly, besides the p38 MAPK signalling pathway, IL-4-induced LOX mRNA appears to be also mediated by the PI-3K pathway, as demonstrated with inhibition of the latter with LY294002. This pathway

universally leads to the activation of Akt mitogenic sequence. As such, the present data are inconsistent with previously documented reports showing that LOX triggers its anti-tumour effects through abrogation of Akt in human lung and pancreatic cancer cells (Wu *et al.* 2007). Nonetheless, whether or not this effect is cell type dependent is not known at this time but it should be noted that functional Akt has been demonstrated in primary, immortalised and neoplastic epithelial ovarian cells with increased expression in EOC (Wong *et al.* 2001). A negative feedback loop mechanism also cannot be excluded. On the other hand, PI-3K involvement has been also shown in IL-4-stimulated 3β-HSD1 and 3β-HSD2 mRNA expression in primary hOSE cells (see Chapter 5) as well as in IL-4-induced 3β-HSD activity of breast cancer cells (Gingras *et al.* 2000), attesting that participation of this pathway in anti-inflammatory activities is possible.

The caveat regarding LOX studies is that we have only assessed transcriptional mRNA regulation of LOX without investigating the effects of cytokines on LOX protein. This is very fundamental given the post-transcriptional and post-translational modifications that LOX undergoes (Ricard-Blum *et al.* 2002, Trackman *et al.* 1992, Uzel *et al.* 2001). For instance, in the osteoblastic MC3T3-E1 cells, it has been established that LOX activity does not correlate with LOX mRNA levels (Feres-Filho *et al.* 1995).

In summary, our data further support the anti-inflammatory nature of IL-4 in the ovarian cell surface. Besides its effects on steroid biosynthesis during post-ovulatory healing, IL-4 also appears to control integral deposition of the ECM through attenuation of IL-1α-induced COX-2 mRNA and also elevation of LOX mRNA in hOSE. Concomitant IL-4-induced progesterone biosynthesis could further contribute in suppression of IL-1α-increased COX-2 mRNA, attesting to a positive feedback loop mechanism. IL-1α- and IL-4-mediated LOX mRNA levels appear to be mediated by a p38 MAPK pathway. In the case of IL-4, an involvement of the PI-3K pathway was also shown. A schematic illustration of the proposed model is given in Fig. 7.5.

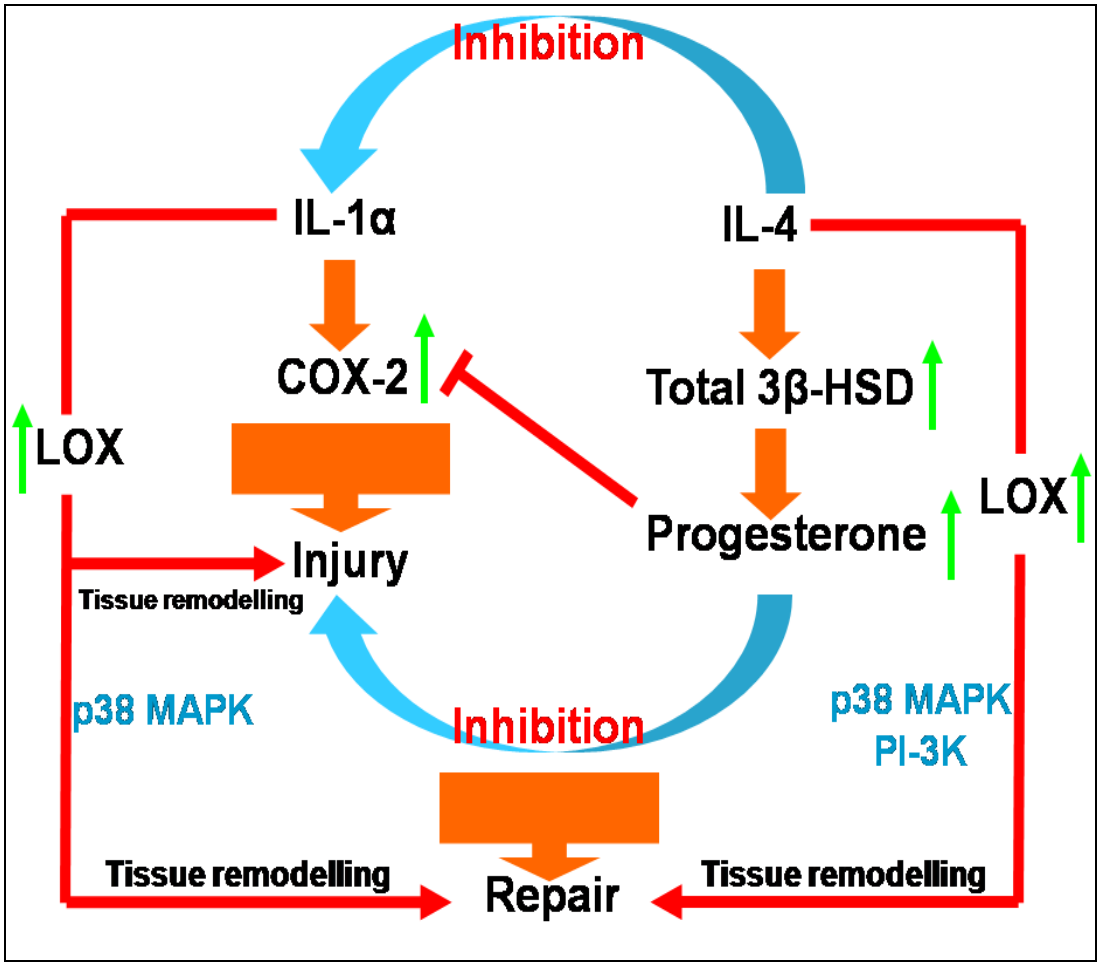


Figure 7.5: Role of IL-4 in tissue remodelling of hOSE. This figure illustrates schematically the effects of IL-4 on COX-2 and LOX mRNA levels in the presence or absence of IL-1α. Similarly to progesterone, IL-4 attenuates IL-1α-induced COX-2 mRNA. Concomitant IL-4-stimulated progesterone biosynthesis might further enhance this inhibitory effect, suggestive of a positive feedback loop mechanism. IL-4-stimulated LOX mRNA further supports its fundamental role in connective tissue deposition during post-ovulatory repair of hOSE. IL-1α and IL-4-mediated LOX mRNA appears to be mediated by p38 MAPK and in the case of IL-4 a cross-talk with PI-3K was also shown.

Chapter 8

Effects of cytokines on 3β-hydroxysteroid dehydrogenases in epithelial ovarian cancer

8.1 Introduction

Ovarian cancer is the most frequent lethal gynaecologic cancer in Europe and North America. It is the 5th most common cause of death after breast, colorectum, lung and endometrial cancer with post-menopausal women at greater risk (Parkin *et al.* 2005). The lifetime risk to develop ovarian cancer is 1 in 70 and only around 30% of the sufferers surviving beyond 5 years (Murdoch & McDonnel 2002, Petricoin *et al.* 2002, Riman *et al.* 2004). The main reason for the high mortality is the asymptomatic progression of the disease along with the absence of efficient screening tests. Also, the lack of proper research models hampers advances in the development of effective prognostic and diagnostic techniques.

As rediscussed in Chapter 1 (Section 1.3), ovarian cancer is classified as a surface epithelial-stromal tumour or EOC when it arises from the OSE, a sex cord-stromal ovarian tumour when it is of mesonephric or mesenchyme origin (granulosa, theca, stromal cells) and a germ cell tumour when it derives from primordial germ cells. The major focus of this Chapter will be on EOC which represents more than 85% of ovarian cancers (Chen *et al.* 2003). Despite the current dogma that EOC arises from the hOSE, novel suggestions regarding the peritoneum surface epithelium and tubal surface epithelium as candidate origins of EOC should not be underestimated (Piek *et al.* 2004). However, the latter is beyond the focus of this thesis.

8.1.1 Classification of epithelial ovarian cancers

As mentioned in Chapter 1, the hOSE is a plastic mesothelium responding to environmental alterations with no commitment to its embryonic origin, the coelomic epithelium (Fig. 8.1A). On the other hand, in neoplastic transformation, cancer cells have a strictly epithelial appearance resembling the Müllerian duct epithelial derivatives (Fig. 8.1B-F).

8.1.1.1 Histological classification of EOC

Based on the World Health Organisation (WHO) classification of ovarian tumours (1973) that was reviewed by the International Agency for Research on Cancer (IARC) in 1998, there are five groups of EOC according to their histological features: serous, mucinous, endometrioid, clear cell and transitional cell or Brenner ovarian tumours. If the tumour does not fall in any of these subtypes, it is characterised as undifferentiated, whereas it is considered as adenocarcinoma not specified (NOS) when it does not have a particular histological morphology (Parkin *et al.* 1998, Scully 1987).

Serous ovarian carcinomas are classified as tumours that resemble the epithelium of the oviduct or fallopian tube (Fig. 8.1B). They usually have multiple cystic chambers with papillary projections to extend out of the surface of the tumour. The diagnosis of these tumours usually occurs at the sixth decade of a woman's life. The serous tumour subtype encompasses almost 50% of the overall ovarian neoplasms.

Mucinous ovarian cancers have features of the uterine cervical or intestinal epithelium (Fig. 8.1C). They contain papillary outgrowths within the cystic chambers with some large solid areas as well. They also display necrotic and haemorrhagic regions. They are also frequent at the sixth decade of life and reflect around 7% of all ovarian tumours.

Endometrioid ovarian tumours feature the glandular epithelium of the endometrium (Fig. 8.1D). They are sometimes associated with endometriosis or endometrial hyperplasia. Although they are quite common among ovarian cancers, they are considered to have a better prognosis than serous or mucinous tumours. They are more usual in sixth decade of life.

Clear cell ovarian neoplasias are characterised by solid, papillary or glandular tumours consisting of cells with clear cytoplasm that contains high levels of glycogen (Fig. 8.1E). They may also appear as cystic masses with polypoids projections. They usually appear in the fifth decade of life more commonly in women who never gave birth. They represent around 6% of all ovarian neoplasms.

Transitional or Brenner cell tumours consist of cells that morphologically look like epithelial cells of the urinary bladder (Fig. 8.1F). They are extremely rare tumours usually diagnosed at an advanced stage. They contain cystic and solid areas with polypoid masses.

EOCs that consist of cells with malignant features such as pleomorphic nuclei and undifferentiated cytoplasms are classified as undifferentiated carcinomas. Such EOC reflect approximately 5% of all ovarian neoplasms and they are commonly diagnosed during the sixth decade of life.

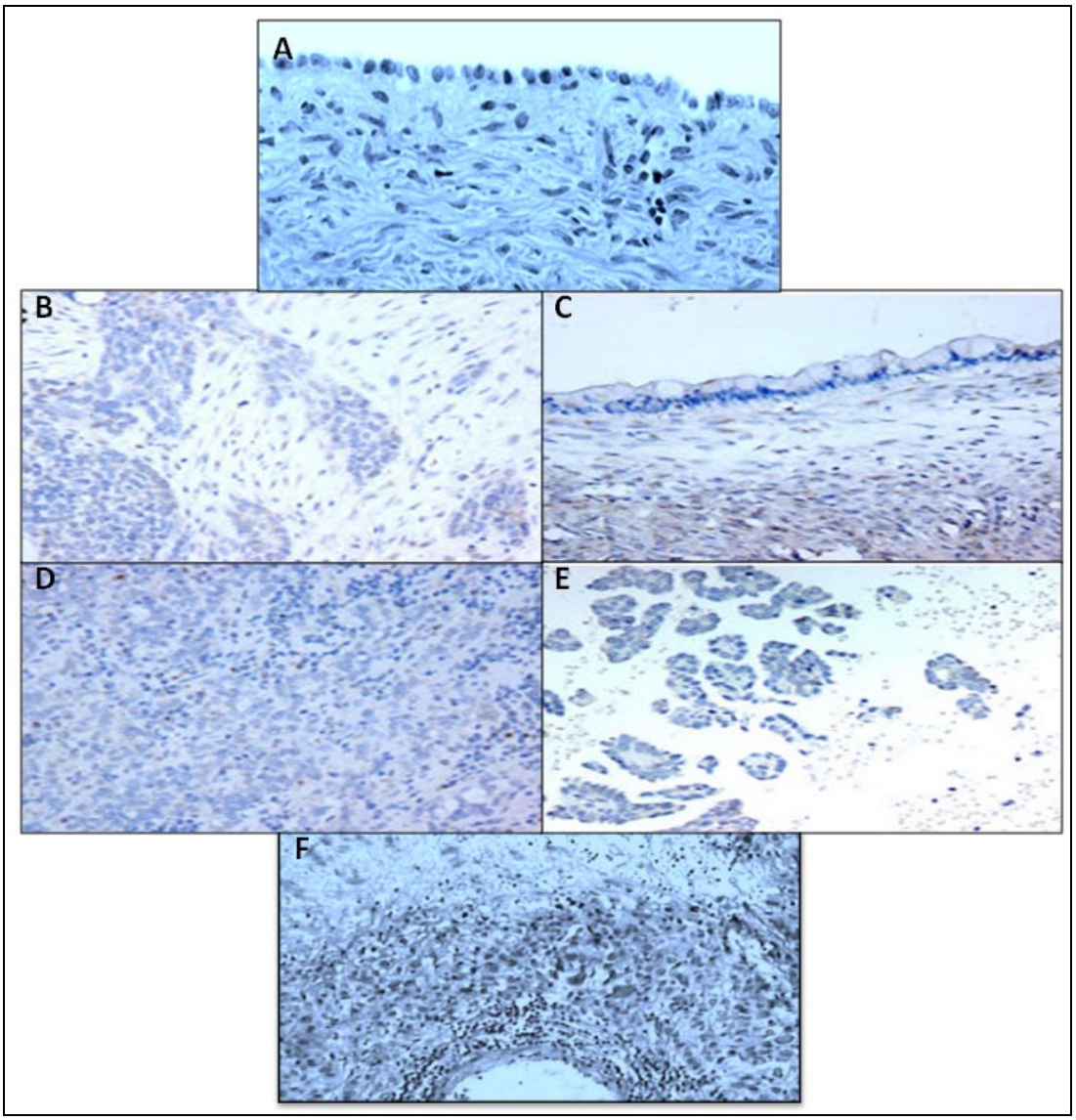


Figure 8.1: Epithelial ovarian cancer histological subtypes. Ovarian epithelial tumours that arise from hOSE (A) are histologically classified in serous (B), mucinous (C), endometrioid (D), clear cell (E) and transitional cell (F) ovarian neoplasms. Adapted from http://www.lmp.ualberta.ca/resources/pathoimages/Images-P/000p0519.jpg (2008) and Nunez *et al.* (2005).

8.1.1.2 Staging and grading of ovarian cancer

Staging of ovarian cancer is conducted through the TNM parametric system (T stands for extent of primary tumour, N stands for degree of spread to lymph nodes and M stands for metastasis). This system was established by the American Joint Committee of Cancer (AJCC) and the International Federation of Obstetrics and Gynaecology (FIGO) (Flemming *et al.* 1997, Kosary 1988). The EOC staging protocol is illustrated in Table 8.1.

The grade (G) of ovarian cancer represents the degree to which the tumour bears a resemblance to the healthy tissue. The grading of the ovarian tumour is of prognostic importance and might prove useful for therapeutic strategies that might benefit the patient. G1 refers to tumours that are well-differentiated, G2 to moderately-differentiated tumours and G3 to poorly-differentiated tumours (Chen *et al.* 2003).

Table 8.1 Staging of ovarian cancer according to AJCC and FIGO

AJCC	FIGO	Tumour Description
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1	I	Limited to one or both ovaries
T1a	IA	Limited to one ovary; capsule intact, no tumour on ovarian surface. No tumour cells in ascities or peritoneal washings
T1b	IB	Limited to both ovaries; capsules intact, no tumour on ovarian surface. No malignant cells in ascites or peritoneal washings.
T1c	IC	Limited to one or both ovaries, with capsule ruptured and/or tumour on ovarian surface and/or malignant cells in ascites or peritoneal washings
T2	II	Tumour involves one or both ovaries with pelvic extension
T2a	IIA	Extension and/or implants on uterus and/or tube(s). No malignant cells in ascites or peritoneal washings
T2b	IIB	Extension to other pelvic tissues. No malignant cells in ascites or peritoneal washings
T2c	IIC	Pelvic extension with malignant cells in ascites or peritoneal washings
T3	III	Tumour involves one or both ovaries, with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis
T3a	IIIA	Microscopic peritoneal metastasis beyond pelvis
T3b	IIIB	Macroscopic peritoneal metastasis (2 cm or less in greatest dimension) beyond pelvis
T3c	IIIC	Peritoneal metastasis (more than 2 cm in greatest dimension) beyond pelvis and/or regional lymph node metastasis
M1	IV	Distant metastasis (excludes peritoneal metastasis)

Adapted from Chen *et al.* (2003)

8.1.2 Hypotheses for the development of EOC and aim of the Chapter

As discussed in Chapter 1, physiological reproductive events are considered to contribute in the development of epithelial ovarian cancer. Ovulation-associated inflammation coupled with production of reproductive hormones including gonadotrophins and steroids pre- and post-ovulatory could contribute in the emergence of an oncogenic phenotype (Cramer & Welch 1983, Fathalla 1971, Ness *et al.* 2000b, Risch 1998). In Chapters 3, 4 and 6 it was shown that functional 3β-HSD activity is possibly a critical event in the maintenance of hOSE homeostasis, intracrinally generating progesterone that activates its cognate PR that in turn exerts its apoptotic (Bu *et al.* 1997) and anti-inflammatory (Rae *et al.* 2004a) effects. Moreover, it was shown that IL-4 sustains local progesterone pre-receptor metabolism and downstream signalling such that integral restoration of the stigma is secured during post-ovulatory healing. This agrees with previous studies illustrating that expression of progesterone-related genes *i.e.* StAR, 3β-HSD and PR in ovarian cancers are related with good prognosis of the disease (Abd-Elaziz *et al.* 2005). On the other hand, elevated levels of androgen-related genes such as AR and AR co-activators are commonly encountered in ovarian cancers (Cardillo *et al.* 1998, Laughlin *et al.* 2000, Shaw *et al.* 2001).

Therefore, given the proposed importance of the functionality of 3β-HSD along with the demonstrated altered expression of the enzyme during injury and repair of hOSE, the objective of this Chapter is to evaluate if the 3β-HSD pathway is defective in epithelial ovarian cancer.

8.2 Subjects and Methods

In order to evaluate 3β-HSD protein expression in EOC, 3β-HSD immunohistochemistry (IHC) of primary ovarian solid tumours as well as freshly isolated ascites from women who were diagnosed with EOC was carried out. Transcriptional mRNA expression levels were also measured for 3β-HSD1 and 3β-HSD2 mRNAs as well as IL-4R and IL-1R1 mRNAs in cultured ascites cells and compared with those of normal cultured hOSE cells. Finally, we performed functional studies where treatments of primary ascites with IL-1α and IL-4 took place followed by Taqman qPCR to determine effects of these proxies on 3β-HSD1 and 3β-HSD2 mRNA species. The basic clinical profile of patients who were used to complete these studies is given in Tables 8.2 and 8.3.

Table 8.2 Clinical profile of patients whose hOSE were used for comparison studies with ovarian cancer cells

Patient No	Code	LREC No	Age (yrs)	Surgery	Reason for surgery	Cycle day/phase	Study
10	5536	04/S1103/36	23	DiagLapar	HMB/pain	n/s	qPCR
11	5537	04/S1103/36	24	DiagLapar	Dysmenorrhoiea	(28) Luteal	qPCR
12	7383	04/S1103/36	43	TAHBSO	Fibroids	n/s	qPCR
13	7384	04/S1103/36	42	TAH	HMB	(23) Luteal	qPCR
20	7423	04/S1103/36	49	TAH	Fibroids	(18) Luteal	qPCR
37	7414	04/S1103/36	22	TAHBSO	Fibroids	n/s	qPCR

TAH: total abdominal hysterectomy, TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy, HMB: heavy menstruation bleeding, DiagLapar: diagnostic laparoscopy, n/s: not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days.

Table 8.3 Pathology of ovarian cancer patients

IHC	Age	Status	Histolgy	CA 125 (units/mL)	Stage	Gra-de
Ascites 1	88	Post-menopausal	Serous/endometrioid	202	IIIB	2
Ascites 2	73	Post-menopausal	Serous	447	IV	3
Ascites 3	66	Post-menopausal	Endometrioid	3101	IV	3
Solid tumor 1	61	Post-menopausal	Papillary serous with minor mixed components	21480	IIIC	3
Solid tumor 2	43	Pre-menopausal	Mucinous/serous/ endometrioid	17912	IIC	3
Solid tumor 3	41	Pre-menopausal	Metastasis from breast	46	*	3
Solid tumor 4	59	Post-menopausal	Poorly differentiated serous	1387	IIIC	3
Solid tumor 5	59	Post-menopausal	Poorly differentiated serous papillary	3067	IV	3
hOSE vs ascites						
Ascites 4	55	Post-menopausal	Papillary serous	2647	IIIC	3
Ascites 5	74	Post-menopausal	Poorly differentiated serous/endometrioid	305	IIIC	3
Ascites 6 (as tumor 3)	41	Pre-menopausal	Metastasis from breast	46	*	3
Ascites 7	67	Post-menopausal	Serous	353	IIIC	3
Ascites 1	88	Post-menopausal	Serous/endometrioid	202	IIIB	2
Ascites 2	73	Post-menopausal	Serous	447	IV	3
IL-1α treatment						
Ascites 7	67	Post-menopausal	Serous	353	IIIC	3
Ascites 1	88	Post-menopausal	Serous/endometrioid	202	IIIB	2
Ascites 2	73	Post-menopausal	Serous	447	IV	3
IL-4 treatment						
Ascites 6	41	Pre-menopausal	Metastasis from breast	46	*	3
Ascites 8	53	Post-menopausal	Serous	447		3
Ascites 2	73	Post-menopausal	Serous	270		3

IHC: immunohistochemistry. Pathological assessment was conducted by Professor Alistair Williams Royal Infirmary of Edinburgh Pathology department. LREC project number: 04/S1103/44.

8.3 Results

8.3.1 Immunolocalisation of 3β-HSD protein in ovarian cancer

8.3.1.1 Immunolocalisation of 3β-HSD protein in solid ovarian tumours

Colourimetric immunohistochemistry was conducted on paraffin-embedded sections (3μm) of ovarian solid tumours from 5 patients (Table 8.3). In all 5 patients, 3β-HSD protein was immunodetected in the ovarian tumours. Cytokeratin 7 (CK 7) staining revealed that 3β-HSD was mainly present in epithelial cells. A representative block is illustrated in Fig. 8.2. Characterisation of this tumour is given in Table 8.3 (solid tumour 1).

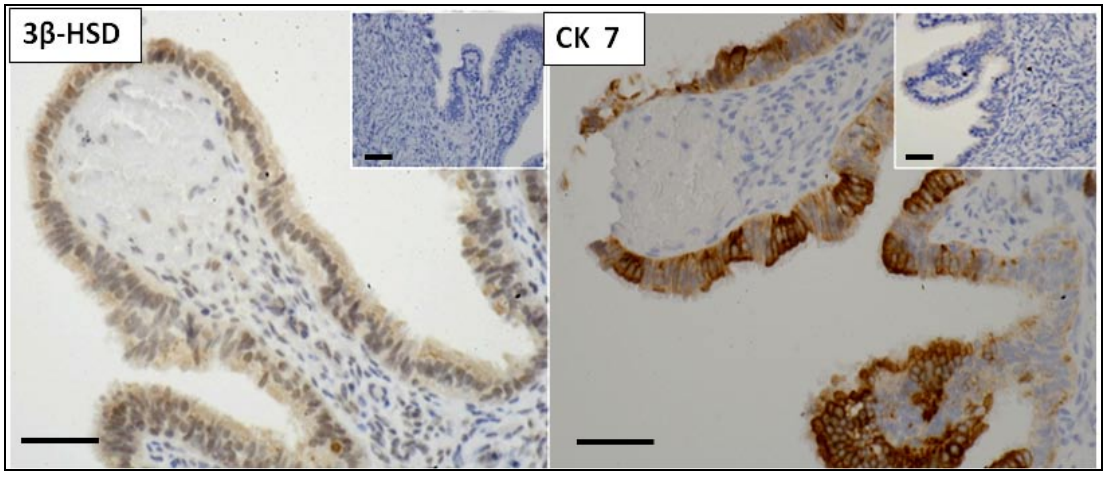


Figure 8.2: Immunodetection of 3β-HSD protein in a representative solid ovarian tumour. Colourimetric immunohistochemistry with the 3β-HSD antibody (1:4000 dilution) showed expression of 3β-HSD protein (left panel) in cytokeratin 7 (CK 7) positive epithelial cells (right panel). Insets represent negative controls for 3β-HSD (left panel) and CK 7 (right panel). Slides were visualised with an Olympus Provis microscope and pictures were captured with a digital 30D Canon camera in 40X magnification. Scale bars represent 50μm.

8.3.1.2 Immunolocalisation of 3β-HSD protein in ascites cells from ovarian cancer patients

Colourimetric immunohistochemistry of freshly isolated ascites for 3β-HSD (1:4000 dilution) and CK 7 (1:1000 dilution) showed similar localisation patterns of the two proteins. A representative section is illustrated in Fig. 8.3A (Table 8.3, ascites 3). In order to separate epithelial cells of cancer origin from active mesothelial or immune cells, double immunofluorescence for cytokeratin 7 and 3β-HSD was applied (Fig. 8.3B). Both CK 7 (green) and 3β-HSD (red) were immunolocalised in the cytoplasm of ascitic clumps (Fig. 8.3B b and 8.3B c, respectively). Also, 3β-HSD was expressed exclusively in epithelial-positive cells and not in other contaminating cells (Fig. 8.3B e, white arrows). Counterstaining was achieved with Dapi staining (1:1000 dilution; blue). The malignant phenotype of ascites cells was confirmed with haematoxylin and eosin staining (H+E). Cells were characterised by pleomorphic nuclei and a high mitotic count (Fig. 8.3B f).

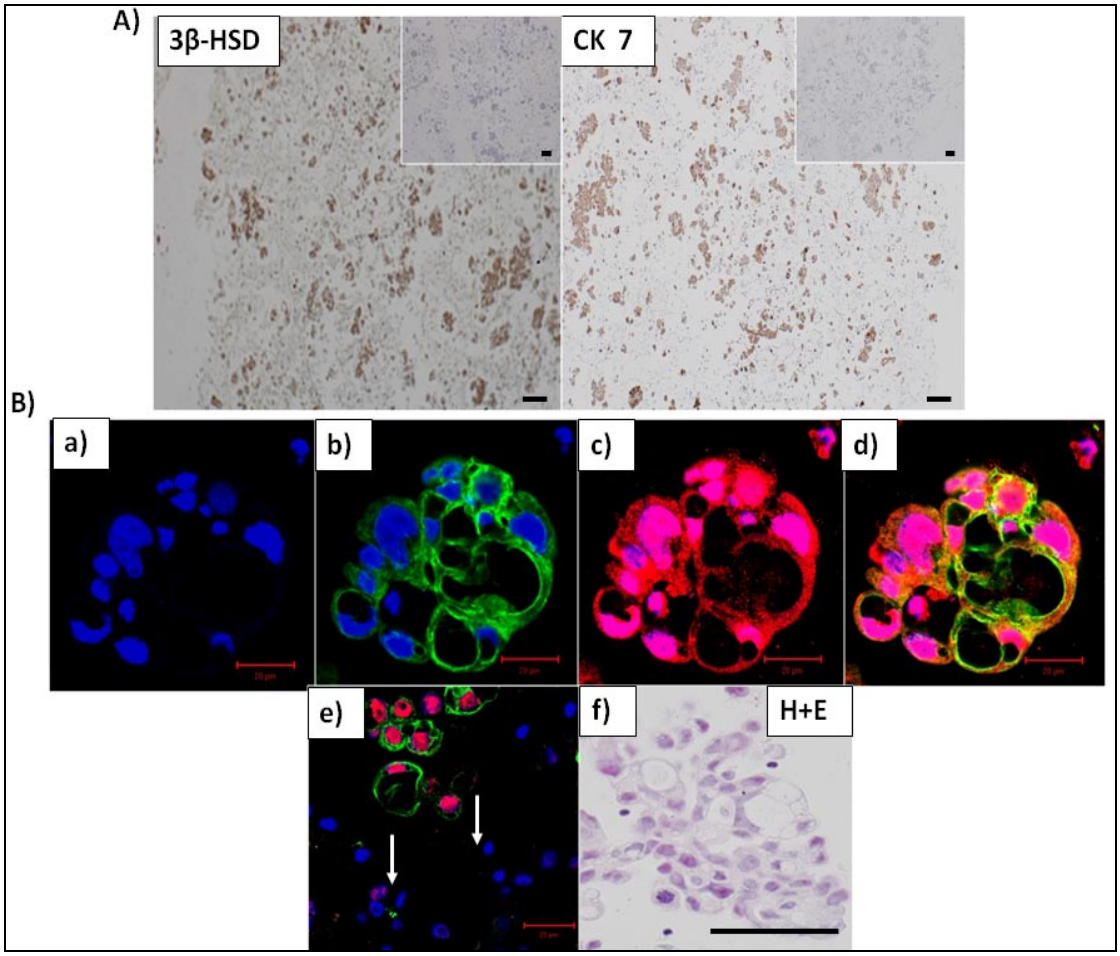


Figure 8.3: Expression of 3β-HSD in freshly isolated ascites. A) Representative slide of colourimetric IHC for 3β-HSD (1:4000 dilution) and CK 7 (1:1000 dilution) in freshly isolated ascites. Insets represent negative controls. Slides were visualised with an Olympus Provis microscope and photomicrographs were captured with a digital 30D Canon camera in 4X magnification. Scale bars represent 200µm. B) Double immunofluorescence for a) Dapi stained nuclei (blue, 1:1000 dilution), b) CK 7 (green, 1:300 dilution, c) 3β-HSD (red, 1:400 dilution), d) merged. Note that 3β-HSD was expressed only in CK 7-positive cells (e). Pictures were captured with a Zeiss LSM 510 meta-confocal microscope in 40X magnification (20µm scale bars). H+E staining of ascites to confirm malignant phenotype of cells (f) (40X magnification, scale bar 50µm).

8.3.2 The expression pattern of 3β-HSD1 and 3β-HSD2 mRNA in ascites from ovarian cancer patients

Because the 3β-HSD antibody immunoreacts with both the 3β-HSD1 and 3β-HSD2 isoforms, we further investigated the isoform expression pattern by qPCR using isoform-specific sets of primers/probes as described in Chapter 2. An assessment of 3β-HSD1 and 3β-HSD2 mRNA species in RNA samples of cultured ascites from 6 patients who were diagnosed with EOC revealed the presence of both transcripts. The mean dCt value for 3β-HSD1 was 21.7, whilst for that of 3β-HSD2 was 24.3. However, increased 3β-HSD1 mRNA levels relative to ones of 3β-HSD2 mRNA were only a trend and did not reach statistical significance (Fig. 8.4).

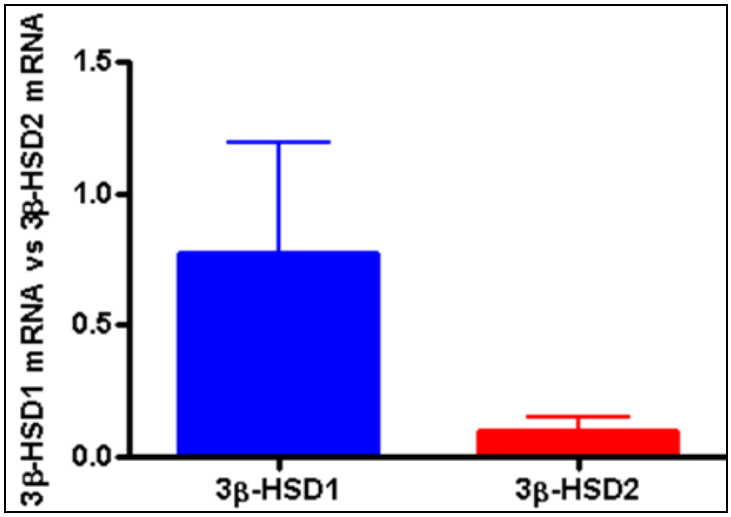


Figure 8.4: Isoform expression pattern in malignant ovarian cells. Taqman qPCR for 3β-HSD1 and 3β-HD2 mRNA in 6 separate primary ascites cell cultures was conducted as described in Chapter 2, section 2.3.4. Measurable transcripts were observed for both transcript species. The mean dCt value for 3β-HSD1 was 21.7, whilst for that of 3β-HSD2 was 24.3.

8.3.3 The relative expression of target genes in primary cultured EOC cells and hOSE cells

8.3.3.1 Relative expression mRNA levels of 3β-HSD1 and 3β-HSD2 mRNA in primary hOSE and EOC cells

After establishment of the presence of 3β-HSD protein and mRNA transcripts in EOC, we determined whether there was any difference in 3β-HSD1 and 3β-HSD2 mRNA species in 6 independent primary hOSE and 6 independent EOC cell monolayers. Taqman qPCR showed that average 3β-HSD1 mRNA levels in hOSE cell samples were 42 times higher than those of EOC cell samples (Fig. 8.5, upper panel, b=p<0.05). On the other hand, 3β-HSD2 mRNA levels in EOC were not statistically different from hOSE cells, although a trend towards down-regulated 3β-HSD2 in EOC was observed (Fig. 8.5, lower panel, p=0.06).

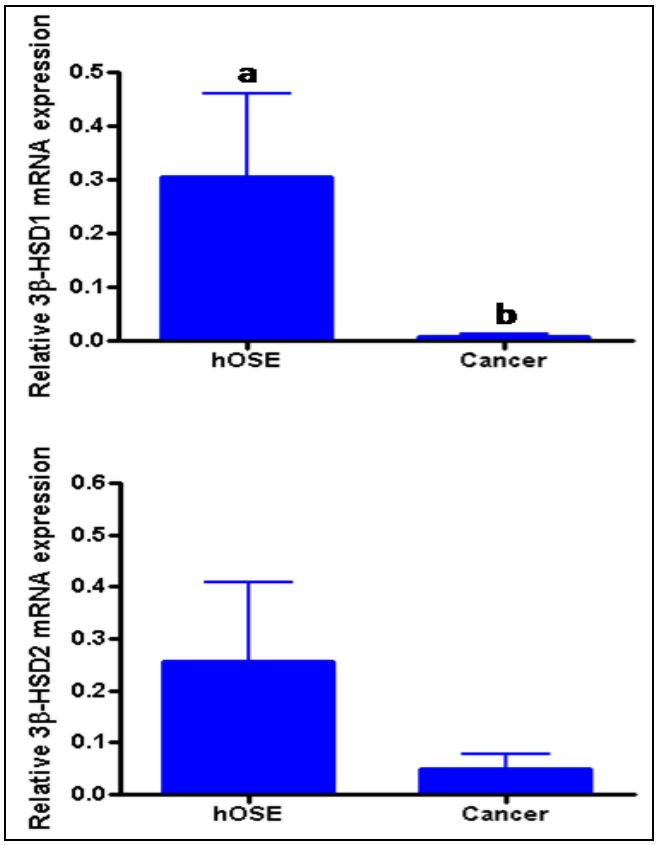


Figure 8.5: Relative 3β-HSD1 and 3β-HSD2 mRNA levels in hOSE and EOC cells. Combined data of 6 separate hOSE and 6 further EOC samples. Taqman qPCR was performed to assess differences in 3β-HSD1 and 3β-HSD2 transcriptional levels between normal hOSE and EOC cells. Mean dCt of 3β-HSD1 mRNA and 3β-HSD2 mRNA in hOSE was 16.3 and 21.6, respectively. In EOC, mean dCt of 3β-HSD1 mRNA was 21.7 and of 3β-HSD2 mRNA was 24.3 (n=6, p=b<0.05).

8.3.3.2 Relative levels of IL-1R1 and IL-4R mRNA in primary hOSE and EOC cells

Prior to functional studies after IL-1α and IL-4 treatments of cultured EOC cells we compared transcriptional levels of the cognate cytokine receptors *i.e.* IL-1R1 and IL-4R mRNA in hOSE and ascites cells. Taqman qPCR in the two groups, each consisting of 6 samples did not show any difference in basal transcriptional levels of either IL-1R1 or IL-4R (Fig. 8.6, upper and lower panels, respectively).

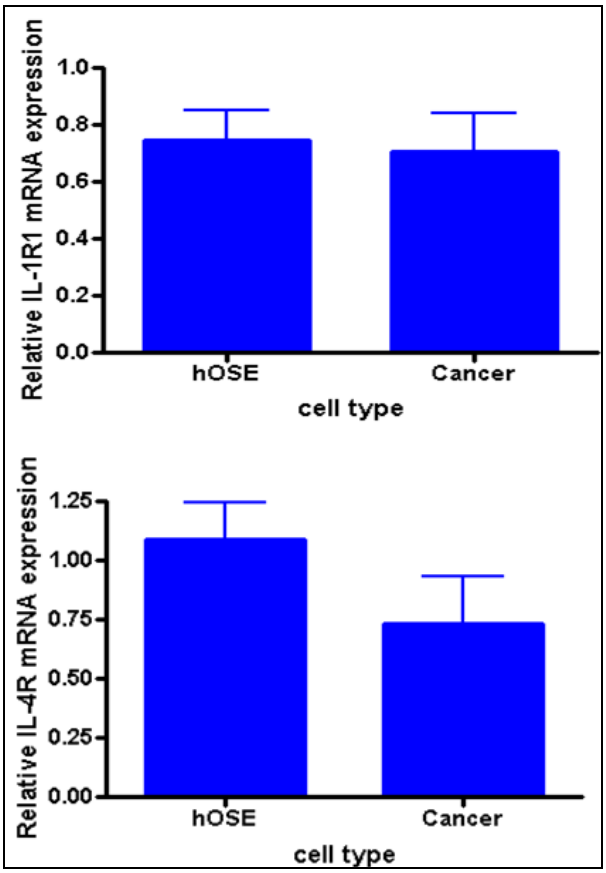


Figure 8.6: Relative IL-1R1 and IL-4R mRNA levels in hOSE and EOC cells. Combined data of 6 separate hOSE and 6 further separate EOC samples. Taqman qPCR was performed to assess differences in IL-1R1 (upper panel) and IL-4R (lower panel) transcriptional levels between normal hOSE and EOC cells. Mean dCt of IL-1R1 mRNA was 13.2 in hOSE and 13.3 in EOC. Mean dCt of IL-4R mRNA was 14.0 and 14.8 in hOSE and EOC, respectively.

8.3.4 Effect of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA levels in primary EOC cells

Primary cultures of ascites collected from 3 patients who were diagnosed with EOC were treated with increasing doses of IL-1α (0.02-1.0ng/mL) for 48h. The cells were then harvested in RNA lysis buffer and after RNA extraction and quantity/quality assay, Taqman qPCR was performed to determine any potential differences in 3β-HSD1 mRNA and 3β-HSD2 mRNA levels between treated and untreated samples. A trend towards an IL-1α down-regulation of 3β-HSD1 mRNA was observed at all doses tested; however, this effect did not reach statistical significance (Fig. 8.7, upper panel, n=3, p=0.058). Regarding 3β-HSD2, there was variability among different patients such that no significant difference was observed between treated and untreated samples although an apparent up-regulatory trend was noted (Fig. 8.7, lower panel, n=3).

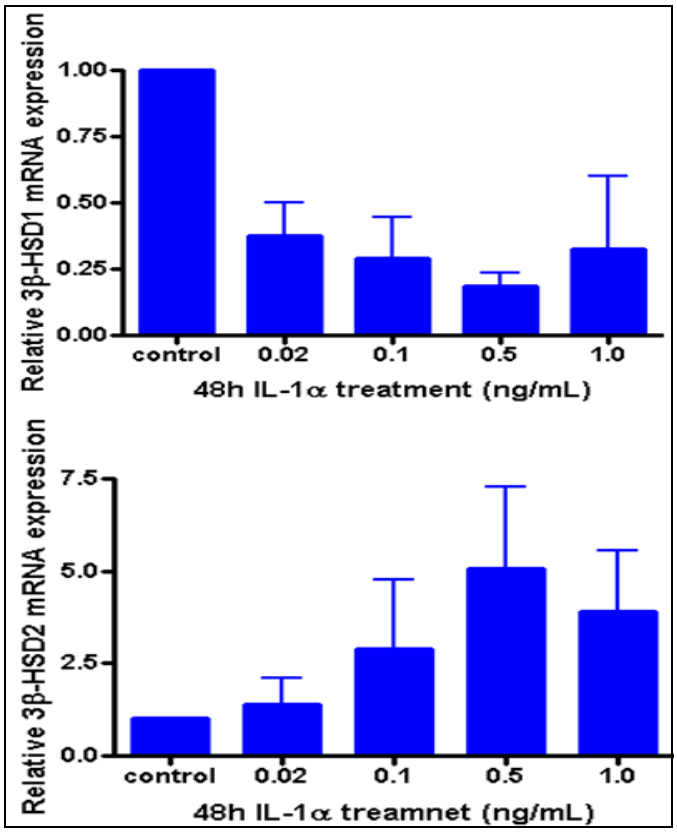


Figure 8.7: Effect of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA in primary EOC cells. Combined data of 3 separate EOC samples. Cells were treated with increasing doses of IL-1α (0.02-1.0ng/mL) for 48h. Taqman qPCR was performed to assess differences in 3β-HSD1 mRNA (upper panel) and 3β-HSD2 mRNA levels (lower panel) between treated and untreated control samples.

8.3.5 Effect of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA levels in primary EOC cells

Primary cultures of ascites collected from 3 patients who were diagnosed with EOC were treated with increasing doses of IL-4 (0.02-1.0ng/mL) for 48h. The cells were then harvested, RNA extracted and quantity/quality assays carried out, Taqman qPCR was performed to determine potential differences in 3β-HSD1 mRNA and 3β-HSD2 mRNA levels between treated and untreated samples. IL-4 substantially increased 3β-HSD1 and 3β-HSD2 mRNA levels when 0.1, 0.5 or 1.0ng/mL IL-4 were added with no statistical difference between different doses (Fig. 8.8, b=$p<0.05$). The average increase of 3β-HSD1 mRNA was approximately 100-fold when 1.0ng/mL IL-4 was added (Fig. 8.8, upper panel), whilst 3β-HSD2 mRNA was increased approximately 80 times by 0.5ng/mL IL-4 (Fig. 8.8, lower panel).

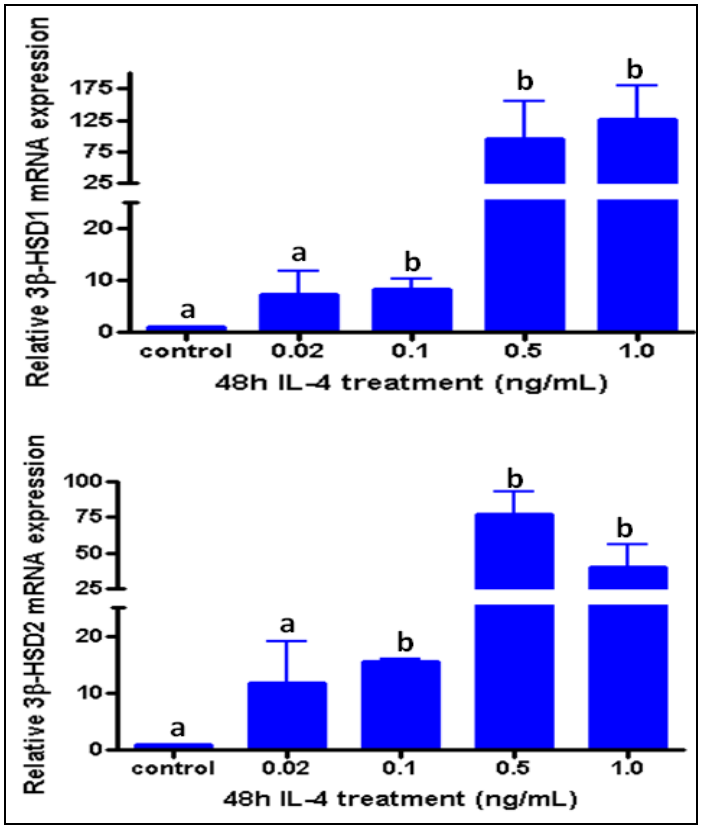


Figure 8.8: Effect of IL-4 on 3β-HSD1 and 3β-HSD2 mRNA levels in primary EOC cells. Combined data of 3 separate EOC samples. Cells were treated with increasing doses of IL-4 (0.02-1.0ng/mL) for 48h. Taqman qPCR was performed to assess differences in 3β-HSD1 mRNA (upper panel) and 3β-HSD2 mRNA levels (lower panel) between treated and untreated control samples (n=3, b=$p<0.05$).

8.3.6 Summary of data accumulated with primary EOC cells

Table 8.4 Comparison studies in primary hOSE and EOC cells

Target gene	Mean dC_T in hOSE	Mean dC_T in EOC	Expression in EOC relative to hOSE
3β-HSD1 mRNA	16.3	21.6	↓
3β-HSD2 mRNA	21.7	24.3	n/s (p=0.06)
IL-1R1 mRNA	13.2	13.3	n/s
IL-4R mRNA	14	14.8	n/s

↓: down-regulation, n/s: not significant; mean dC_T represents PCR cycle when target gene started to accumulate relative to internal 18S. Differences in dC_T between different samples are presented at the logarithmic level

Table 8.5 Effects of IL-1α and IL-4 on 3β-HSD1 mRNA and 3β-HSD2 mRNA in primary EOC cells

Treatment	3β-HSD1 mRNA	3β-HSD2 mRNA
IL-1α	n/s (p=0.058)	n/s
IL-4	↑	↑

↑: up-regulation, n/s: not significant

8.4 Discussion

We have demonstrated 3β-HSD gene expression and enzymatic activity in EOC, suggestive of an intracrine role for 3β-HSD activity in determining functional cellular responses mediated by AR and PR in ovarian cancer. We also have established that both 3β-HSD1 and 3β-HSD2 mRNAs are expressed in EOC. However, we found that ovarian cancer cells have much lower levels of overall 3β-HSD gene expression, but importantly that this can be restored by exposure to IL-4.

The finding that 3β-HSD is expressed in solid ovarian tumours and epithelial cells of malignant origin implies that ovarian tumours have the capacity locally to generate active progestogens and androgens. Importantly, in double immunofluorescence studies of ascites samples, it was shown that only epithelial cytokeratin 7-immunopositive cells expressed 3β-HSD. This suggests that our 3β-HSD data for primary ascitic cell cultures most likely reflect malignant epithelial cells rather than other cellular components of the cultures such as activating mesothelial cells. Notwithstanding this potential limitation, comparisons of ascites with normal hOSE cells established that 3β-HSD mRNA expression is substantially reduced in ovarian cancer cells. Collectively, these data are suggestive of attenuated pre-receptor metabolism in cancer cells relative to normal hOSE, which might be a feature of neoplastic transformation.

A caveat to our comparison of normal hOSE and ovarian cancer cells is that we have compared pre-menopausal normal hOSE with post-menopausal cancer cells, instead of post-menopausal normal hOSE with cancer. Indeed, the differences in 3β-HSD expression levels observed herein might reflect a feature of the post-menopausal ovary in general rather than a feature of oncogenesis, although the study of a single pre-menopausal cancer patient did not show any difference in 3β-HSD expression levels relative to post-menopausal cancers (Table 8.3, ascites 6). However, this finding remains important because attenuation of progesterone biosynthesis post-menopausally could lead to defects of apoptotic molecular mechanisms that underlie

progesterone signalling. This, in turn, could result in tumourigenesis (Bu *et al.* 1997, Murdoch *et al.* 2001). On the other hand, 3β-HSD attenuation in post-menopausal and ovarian cancer women might be considered beneficial since minimisation of local androgen synthesis could suppress cell growth and survival (Edmondson *et al.* 2002). Nevertheless, this is likely not the case since it is well established that the post-menopausal ovary retains the steroidogenic capacity to produce androgens (Havelock *et al.* 2006, Laughlin *et al.* 2000), suggesting alternative pathways rather than 3β-HSD for local androgen production. Previous studies have consistently shown increased AR protein (Cardillo *et al.* 1998, Kühnel *et al.* 1988) and co-activators (Shaw *et al.* 2001) in ovarian cancer, whilst greatly decreased PR mRNA and protein in a significant percentage of ovarian cancers has also been reported (Cardillo *et al.* 1998, Chadha *et al.* 1993, Lau *et al.* 1999). Importantly, the expression of PR in ovarian tumours has been positively associated with an increased survival rate (Abd-Elaziz *et al.* 2005, Hempling *et al.* 1998). Jointly, the shift from a progestogenic to an androgenic-dominant ovarian environment peri- and post-menopausally is probably the consequence of diminished 3β-HSD at this stage of life and could be reflective of the promotion of ovarian cancer.

Because the 3β-HSD antibody cross-reacts with both isoforms, semi-quantitative and quantitative RT-PCR were performed to further elucidate the expression pattern of *HSD3B* gene products (*i.e.* 3β-HSD mRNA). Similarly to hOSE, both mRNA species were present in cultured ascites cells of malignant origin, attesting that both 3β-HSD isoforms likely contribute to overall 3β-HSD enzymatic activity. However, unlike in hOSE cells, there was no statistical difference between 3β-HSD1 mRNA and 3β-HSD2 mRNA levels, although a trend towards increased 3β-HSD1 mRNA was apparent. This was probably reflective of the disparity existing among individual ovarian cancers coupled with a relatively small group of samples.

In Chapters 4 and 5, we distinguished the relative functional importance of 3β-HSD1 and 3β-HSD2 mRNA species in primary hOSE cells. We showed that exposure of hOSE to the pro-inflammatory IL-1α attenuated 3β-HSD1 mRNA

expression, whereas IL-1α stimulated 3β-HSD2 mRNA levels. We also showed that these effects are mediated through differential signalling pathways. In primary cancer cells, 3β-HSD1 mRNA was attenuated by IL-1α treatment (p=0.058), whilst 3β-HSD2 mRNA levels were unaltered by this proxy, suggesting that IL-1α responses to ovarian cancer are altered relative to normal hOSE cells. Nonetheless, there was not any difference in IL-1R1 mRNA expression levels between the two cell populations. Jointly, these findings indicate that altered actions of IL-1α on 3β-HSD gene regulation are not a consequence of lack of IL-1R1 but of changes in downstream signalling molecules. Importantly, a lack of IL-1α to up-regulate 3β-HSD2 mRNA probably represents aberrant local anti-inflammatory steroid action in ovarian cancer. This is consistent with a previous study, demonstrating differences in inflammatory responses between primary hOSE cells and EOC cell lines (Gubbay *et al.* 2005).

The low levels of 3β-HSD expression in ovarian cancer cells and the striking restoration of 3β-HSD1 and 3β-HSD2 mRNA levels after IL-4 treatment of cells of malignant origin, is consistent with an anti-tumourigenic role for this cytokine in ovarian cancer. We note that an anti-tumour role of IL-4 in breast cancer has been also demonstrated (Gingras *et al.* 1999, Nagai & Toi 2000). IL-4 has been reported to attenuate expression of TNF-α- and IL-1α-related pro-inflammatory effects in TNF-α transgenic mice (Bessis *et al.* 1998). Like hOSE cells, cancer cells do not secrete IL-4 *de novo* but they do express the IL-4 receptor (Burke *et al.* 1996). Importantly, we did not observe any differences in IL-4R mRNA expression levels between cancer and hOSE cells, suggesting that it is the absence of the ligand and not the receptor that is associated with tumourigenesis. Consistently, serum IL-4 levels have been shown to be reduced in post-menopausal women (Kumru *et al.* 2004), a stage of life where EOC is commonly encountered. It is very possible therefore, that IL-4-increased 3β-HSD that favours local progesterone signalling in hOSE is lost after the menopause. This could have profound implications for the development of post-menopausal ovarian cancer and deserves further investigation. Moreover, it has been reported that oestrogens can increase interferon-γ levels (Th-1 response), resulting in a lack of an increase in serum IL-4 levels (Th-2 response) (Kumru *et al.* 2004). It is

therefore of interest that a positive correlation exists between oestrogen levels and ovarian cancer (Choi *et al.* 2001b, Mukherjee *et al.* 2005).

In summary, we have demonstrated attenuated expression and functionality of 3β-HSDs in EOC cells relative to normal hOSE. However, IL-4 can stimulate 3β-HSD expression levels, potentially triggering steroid production in the ovarian milieu. IL-4-increased 3β-HSD could therefore be a critical determinant of progesterone bioavailability and anti-tumourigenic signalling via PR with the potential application to the treatment of ovarian cancer. A schematic illustration of our findings is presented in Fig. 8.9.

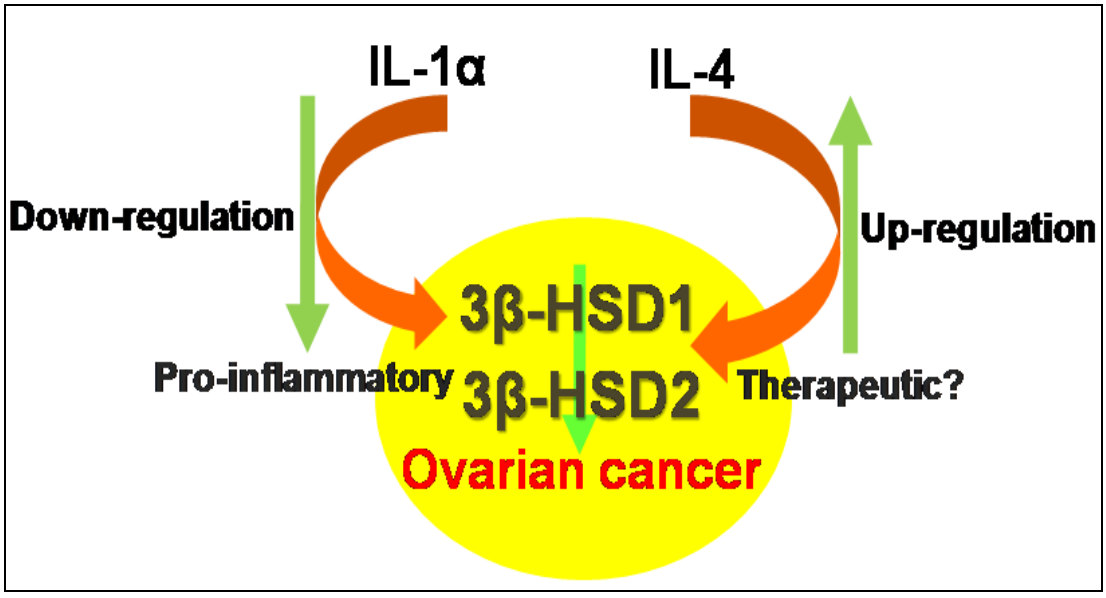


Figure 8.9: Effects of cytokines in EOC. Basally decreased levels of 3β-HSD1 mRNA in EOC is further attenuated in response to IL-1α. However, restoration can be achieved in the presence of IL-4. Also, 3β-HSD2 mRNA can be substantially increased by IL-4 treatment. Collectively, IL-4 stimulatory effects on 3β-HSDs might well be a therapeutic advantageous modality.

Chapter 9

Synopsis of thesis and future directions

9.1 Synopsis and General Discussion

This thesis explored the steroid mechanisms that physiologically occur in the human ovarian surface epithelium (hOSE), a poorly understood compartment of the biology of the ovary. The main focus was the regulation of pre-receptor metabolism of cytoproliferative androgens and apoptotic and anti-inflammatory progestogens, through investigation of 3β-HSD gene and protein function, during injury and repair of the post-ovulatory hOSE. Some preliminary work on cognate steroid androgen (AR) and progesterone (PR) receptors was also performed. Furthermore, as dysfunction of hOSE is also relevant to the development of epithelial ovarian cancer (EOC), preliminary approaches on the 3β-HSD pathway in EOC was also performed.

The study has established that 3β-HSD is localised in the ovarian cell surface, indicating that this ovarian compartment can actively participate in steroid biosynthesis not only of the surface epithelium but also of the whole ovarian tissue at least up to the progesterone level. It was also demonstrated that 3β-HSD is differentially regulated during the post-ovulatory injury and repair of the hOSE, attesting to its significance in tissue homeostasis. Ovulation-associated injury was mimicked with pro-inflammatory cytokines, namely IL-1α, TNF-α, IL-6, IL-18 and GM-CSF, whereas T lymphocyte-associated cytokines, namely IL-4 and IL-10 were used to induce post-ovulatory healing.

In post-ovulatory wounding, the two 3β-HSD isoforms, 3β-HSD1 and 3β-HSD2, were shown to be differentially regulated by IL-1α but little or no effect on total 3β-HSD protein and activity was noticed, within the time window applied, revealing that a balance in steroid biosynthesis is essential for restriction of tissue damage that might arise from ovulation-associated inflammation. Importantly, our studies demonstrated that opposing effects of IL-1α on 3β-HSD1 and 3β-HSD2 mRNA species are a result of activation of different signalling networks; inflammatory NF-κB pathway appears to regulate the IL-1α attenuation of 3β-HSD1 mRNA, whilst a cross-talk among NF-κB, PI-3K and p38 MAPK pathways appears

to involve IL-1α-stimulated 3β-HSD2 mRNA. Moreover, IL-1α did not alter AR or PR mRNA expression levels, further supporting a balance in steroid actions via nuclear receptors during ovulation.

A substantial increase of 3β-HSD1, 3β-HSD2 mRNA and total 3β-HSD protein and activity was established after *in vitro* treatment of hOSE cells with IL-4. Importantly, IL-4-induced 3β-HSD1 and 3β-HSD2 mRNA required activated PI-3K and STAT-6 pathways. Moreover, IL-4 induction of 3β-HSD2 mRNA was suppressed when the SB203580 p38 MAPK pathway inhibitor was added. IL-4 treatment also attenuated AR mRNA and protein without any alteration of PR mRNA expression levels. Intriguingly, this effect involved p38 MAPK signalling pathway. Collectively, it was shown that IL-4 sustains apoptotic and anti-inflammatory progesterone pre-receptor metabolism and signalling via cognate PR, implying an anti-inflammatory role of this cytokine in post-ovulatory healing. To further confirm that IL-4 plays a role in hOSE post-ovulatory repair, we also tested two genes that play a key role in extracellular matrix (ECM) integrity, a process fundamental for proper hOSE regeneration; namely, the pro-inflammatory COX-2 that is responsible for prostaglandin synthesis and thus ECM degradation and LOX that is responsible for ECM deposition. The T lymphocyte-associated cytokine IL-4 was demonstrated to suppress IL-1α-induced COX-2 mRNA and also to stimulate LOX mRNA, thereby implying that this cytokine is essential not only for controlled proliferation of hOSE (through increase of 3β-HSD and thus intracrine generation of progesterone) but also for deposition of ECM. Remarkably, IL-4-induced LOX was abrogated when inhibitors for p38 MAPK and PI-3K pathways were added.

p38 MAPK was clearly shown to be involved in the anti-inflammatory effects of IL-4 and also in the anti-inflammatory effects of IL-1α such as 3β-HSD2 induction. However, as shown by numerous studies that were acknowledged in this thesis, an IL-1-induced p38 MAPK pathway is involved in the regulation of pro-inflammatory-associated processes such as IL-6, IL-8 and prostaglandin synthesis. This raises the question of how p38 MAPK could be targeted pharmacologically to

alleviate rather than trigger inflammatory environments of inflammation-associated diseases such as ovarian cancer. This is more difficult, however, at the case of IL-1α, as this stimulus appears to utilise this pathway for both inflammatory and anti-inflammatory-associated mechanisms; on the one hand, it down-regulates 3β-HSD1 mRNA (pro-inflammatory effect), whereas on the other hand it up-regulates 3β-HSD2 and 11β-HSD1 mRNA, thereby favouring in hOSE anti-inflammatory progesterone and glucocorticoid generation, respectively. On the other hand, IL-4 appears to positively regulate anti-inflammatory genes (3β-HSD1, 3β-HSD2, LOX) and negatively regulate genes that are involved in cytoproliferation (AR) and inflammation (IL-1α-induced COX-2). Intriguingly, all of these genes appeared to be targets of p38 MAPK signalling cascades, at least at the mRNA transcriptional/stabilisation level, suggesting that targeting this pathway through IL-4 rather than IL-1α could be an efficient pharmaceutical strategy in ovarian disorders such as cancer.

Preliminary studies showed attenuated 3β-HSD gene expression in EOC. In primary EOC cell monolayers, it was demonstrated that 3β-HSD1 mRNA was expressed 42 times lower than that of primary hOSE cells. Moreover, a mean 6-fold decrease of 3β-HSD2 mRNA was observed to primary EOC compared to primary hOSE, although this effect did not reach significance ($p=0.06$). Importantly, IL-4 treatment restored attenuated 3β-HSD1 and 3β-HSD2 mRNA and this could be of therapeutic advantage.

9.2 Advantages, Caveats and Future Directions

A primary advantage of our studies was that all experimental sets were performed with primary cells collected from various patients, thereby taking into consideration variability among individual subjects. Therefore, data collected and conclusions drawn could reflect in part *in vivo* conditions. However, restrictions in the availability and numbers of primary cells did not allow us to design extended experiments, thereby many times excluding important parameters such as incubation time- and dose-points of target agents. Moreover, most of the data obtained were

limited at mRNA levels, making our studies one-dimensional. Though, this could be overcome by the use of proper controls, sufficient replicates and a reasonable amount of accumulating data.

Therefore, in order to further take forwrad speculations and conclusions developed in the present thesis, a series of additional studies are essential. Regarding intracellular signalling pathways, it is important to document phosphorylation of target proteins, namely p38 MAPK, NF-κB, PI-3K and STAT-6 by IL-1α and/or IL-4. Also, to further confirm whether IL-1α and IL-4 actions are antagonistic or independent, co-treatment experiments should be pursued in order to evaluate their combined effects on NF-κB and p38 MAPK signalling pathways. Importantly, it should also be ascertained that the pathway inhibitors tested specifically block phosphorylation of those proteins. Moreover, it would be quite conclusive to investigate which of the pathway inhibitors affect protein levels besides their impact on transcription/mRNA stabilisation of target genes. Also, given the importance of pre-receptor generation of progesterone in hOSE, it is fundamental to identify additional agents that positively or negatively regulate its generation. This could also contribute to the development of efficient strategies to diagnose or treat ovarian disorders such as cancer.

Preliminary studies in primary EOC cells demonstrated that 3β-HSD gene expression was decreaseed in the malignant ovary but this attenuation could be counteracted by treatment with IL-4. Attenuation in overall 3β-HSD protein and activity in EOC relative to hOSE followed by restoration thereof by IL-4 should be also shown to fundamentally establish this observation. It would be also of value to investigate if these effects are suppressed by addition of pathway inhibitors as shown in primary hOSE cells.

It is apparent that limitations in primary tissue availability and cell numbers yielded from one specimen hampers extended exploration of the human ovarian cell surface along with its role in the development of EOC. It is therefore clearly necessary to identify proper animal and human cell line models that could be used to

advance more consistently the biology of the human ovarian surface epithelium and also of human ovarian cancer.

9.3 General Conclusion

In the present thesis, we demonstrated a functional 3β-HSD pathway in the ovarian cell surface that is differentially regulated during injury and repair of post-ovulatory hOSE. Importantly, we showed attenuated expression and functionality of 3β-HSDs in EOC cells relative to normal hOSE, probably reflecting an acquired feature of neoplastic transformation of hOSE. However, IL-4 could stimulate 3β-HSD expression levels, potentially triggering steroid production in the ovarian milieu. IL-4-increased 3β-HSD could therefore be a critical determinant of progesterone bioavailability and anti-tumourigenic signalling via PR during ovulatory menstrual cycles with potential application to the treatment of ovarian cancer. We also identified p38 MAPK signalling pathway as a fundamental regulator of these processes with prospective use of this pathway to target inflammation-associated disorders of the ovary including epithelial ovarian cancer.

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Appendix 1

RNA extraction using Qiagen RNeasy mini-kit (West Sussex, UK)

During the whole procedure, centrifugations were run for 15sec at 5000 x g, followed by discard of the flow-through, unless otherwise indicated. Homogenised lysates were incubated at 37^{o}C for 20min. 0.35mL of 70% ethanol was added to each cell homogenate and the resultant 0.7mL volumes were transferred to RNeasy mini columns placed on collection tubes. Then, DNase digestion was conducted using the Qiagen RNase-Free DNase set. Briefly, 0.35mL RW1 (washing buffer) was added followed by centrifugation. Then, 0.08mL DNase I mix, containing 0.01mL DNase I enzyme diluted in 0.07mL buffer RDD, was added to each column and lysates were centrifuged after incubation for 15min at room temperature. Finally, columns were washed with RW1 and centrifuged. For the RNA isolation, mini columns were washed with 0.5mL of RPE buffer twice (washing buffer). Two centrifugation steps followed. The second centrifugation lasted 2min in order to dry the silica-gel membranes. Then, mini columns were centrifuged for 1min at full speed. For the elution of the RNA, 0.03mL RNase-free water was added to each column placed on a new 1.5mL collection eppendorf, and the RNA was eluted after centrifugation for 1min. To increase the RNA concentration, the eluate RNA was pipetted directly onto silica-gel membranes and centrifuged again for 1min. RNA was stored at -70^{o}C until further use.

Appendix 2

Presentation of work described in that thesis in scientific meetings

Appendix 2

POSTER PRESENTATIONS

- **15-18 June 2008, San Frasisco, CA: 90th Annual Meeting of Endocrine Society**

Steroid signalling in human ovarian surface epithelium wound-healing (Trainee poster competition winner in Steroid hormone biosynthesis and metabolism).

- **7-8 March 2008, London, UK: 1st International Meeting of Ovarian Cancer Action**

Evidence for the attenuation of progesterone biosyntheis in primary cultures of epithelial ovarian cancer. Could IL-4 have a therapeutic role?

- **11-12 September, 2007, Edinburgh, UK: MRC Inflammation Showcase**

3β-Hydroxysteroid dehydrogenase type1 and type 2 in the human ovarian surface epithelium: Differential regulation and transduction of the two isoforms by cytokines

- **2-5 June, 2007, Toronto,Canada: The Endocrine's Society 89th Annual Meeting**

3β-Hydroxysteroid dehydrogenase type1 and type 2 in the human ovarian surface epithelium: Differential regulation and transduction of the two isoforms by cytokines (Awarded to attend ENDO trainee day).

- **13-16 September, 2006, Athens, Greece: 12th International Congress on Hormonal Steroids and Hormones & Cancer**

3β-Hydroxysteroid dehydrogenase type1 in the human ovarian surface epithelium: Down-regulation by pro-inflammatory cytokines (Top 10 poster prize for young investigators).

Appendix 2

ORAL PRESENTATIONS

- **13th November 2007, CRB Research Day**

Regulation of 3β-hydroxysteroid dehydrogenases (3β-HSDs) by cytokines

- **8-9 September, 2007, Cambridge, UK: 9th National Ovarian Workshop**

3β-Hydroxysteroid dehydrogenase type 1 and type 2 in the human ovarian surface epithelium: Differential regulation and transduction of the two isoforms by cytokines

- **11th May, 2007, Aberdeen, UK: Scottish Society of Experimental Medicine**

Altered expression of 3β-hydroxysteroid dehydrogenase type 1 and type 2 among the human ovarian surface epithelium (hOSE) cells, pre-neoplastic and cancer cell lines: Regulation by inflammatory and anti-inflammatory agents

Appendix 3

Peer-reviewed publication in Molecular and Cellular Endocrionology

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3β-Hydroxysteroid dehydrogenases and pre-receptor steroid metabolism in the human ovarian surface epithelium

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ABSTRACT

Ovulation-associated inflammation with accompanied cytokines and reproductive hormones impact upon the human ovarian surface epithelium (hOSE) and probably have a role in the aetiology of ovarian cancer. Progesterone and progestin-related events, *i.e.* pregnancy and oral contraception, protect from the disease. We have investigated the pre-receptor metabolism of progesterone in primary hOSE cells and an immortalised hOSE cell line, OSE-C2, focusing on transcriptional regulation of 3β-hydroxysteroid dehydrogenase (3β-HSD) by inflammatory, anti-inflammatory and apoptotic factors. In hOSE cells, we show that anti-inflammatory effects of IL-1α and IL-4 on 3β-HSD2 mRNA involve a p38 MAPK signalling pathway, whereas pro-inflammatory response of IL-1α to 3β-HSD1 mRNA involves a NF-κB inflammatory pathway. In OSE-C2 cells, retinoic acid and transforming growth factor-β1 massively induce 3β-HSD1 mRNA levels. In conclusion, we elaborate several mechanisms for intracrine formation of progesterone in hOSE that could contribute in the development of novel strategies to prevent, diagnose and/or treat ovarian cancer.

1. Introduction

The human ovarian surface epithelium (hOSE) is a squamous-to-cuboidal cellular monolayer of mesodermal origin that covers the ovary (Gillett et al., 1991). It was only lately that it has been realised that the human surface epithelium is probably the origin of at least 85% of ovarian cancers, the so-called epithelial ovarian cancer (EOC), with stromal-sex cord and germ cell ovarian tumours only representing sparse cases (Parkin et al., 2005). At least three hypotheses have been reported to explain the development of EOC that are interrelated and not mutually exclusive. Ovulation, accompanied inflammation and associated reproductive hormones (*i.e.* gonadotrophins, oestrogens, androgens, progesterone) prior to and post-ovulation are believed to have a role in the aetiology of EOC (Cramer and Welch, 1983; Espey, 1994; Fathalla, 1971; Ness and Cottreau, 1999; Risch, 1998). In essence, the ovarian cell surface undergoes injury and repair cycles after each ovulation and factors such as gonadotrophins, steroid hormones and immune mediators that are released at this time are primary candidates for the progression of or protection from the disease. A series of studies focusing on anti-inflammatory steroid signalling on hOSE led to the concept that identification of anti-inflammatory steroid mechanisms that essentially control integrity of the ovarian cell surface after ovulation-associated wounding could undoubtedly lead to the development of efficient strategies to diagnose and/or treat EOC (Rae and Hillier, 2005; Rae et al., 2004b).

From a recent screening of steroid-related markers in hOSE, it was shown that 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1) mRNA is suppressed by interleukin-1α (IL-1α), an ovulation-associated pro-inflammatory proxy (Rae et al., 2004b). 3β-HSD1 and 3β-HSD2 are two separate isoforms of the same enzyme (they arise from different genes) that control the intracrine formation of progesterone. Progesterone has been reported to be apoptotic and anti-inflammatory in hOSE and EOC cells and links of pregnancy, oral contraception (especially progestin-only contraception) and reduced risk of emergence of the disease have been demonstrated (Bu et al., 1997; Murdoch and Van Kirk, 2002; Rae et al., 2004a; Risch et al., 1994; Rodriguez et al., 1998). 3β-HSD1 is mainly expressed in trophoblast, mammary and skin, whilst 3β-HSD2 is principally expressed in steroidogenic organs such as gonads and adrenal (Lorence et al., 1990; Rheaume et al., 1991; Thomas et al., 1988, 1989). Therefore, although the relevant contribution of each subtype in total 3β-HSD activity cannot be

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predicted, total 3β-HSD activity essentially controls the bioavailability of progesterone and its access to progesterone receptor (PR) in a tissue. Therefore, it is of great importance to elucidate the mechanisms that are involved in progesterone pre-receptor metabolism in hOSE. In support of this, characterisation of inflammatory, anti-inflammatory and apoptotic mediators that could potentially mediate progesterone pre-receptor metabolism should be a priority. For example, IL-1α is a prototypic cytokine that is secreted during ovulation and induces changes in a series of inflammatory-associated genes (Rae et al., 2004a,b). Additionally, IL-4 is a T-lymphocyte cytokine that is mainly secreted at the periovulatory follicle, corpus luteum and corpus luteum of pregnancy (all characterised by elevated progestogens) (Hashii et al., 1998). Another candidate could be transforming growth factor-β1 (TGF-β1) that has been demonstrated to have a role in ovarian tissue remodelling and more importantly has been established to exert anti-proliferative and apoptotic effects on hOSE and EOC (Berchuck et al., 1992; Choi et al., 2001; Harlow et al., 2003). Finally, retinoic acid (RA), a derivative of vitamin A, is considered to be chemopreventive and anti-carcinogenic and has been shown to exert apoptotic effects in EOC cell lines (Bu et al., 1997; Formelli and Cleris, 1993). Remarkably, aldehyde dehydrogenases (ALDH), the enzymes that are involved in oxidation/reduction of retinol and retinal as well as retinoic acid receptors (RAR) are present in hOSE cells, suggesting functional signalling by retinoic acid in hOSE physiology (Rae et al., 2004b).

Limitations in primary tissue availability do not allow sustained investigation of hOSE physiology, a restriction that literally harnesses good prognosis of EOC. As such, identification of models to study hOSE biology appears essential for prevention and/or diagnosis of the disease. OSE-C2 cell line has been established by immortalisation of hOSE cells obtained from a pre-menopausal woman undergoing surgery for non-malignant gynaecological conditions (Davies et al., 2003). The advantage of this cell line is that it maintains its the epithelial-specific cytokeratin staining pattern (Davies et al., 2003), characteristic of primary hOSE cells in tissue culture (Auersperg et al., 2001; Dyck et al., 1996; Rae et al., 2004b). Finally, the OSE-C2 cell line is not considered cancerous, because it does not express E-cadherin, a cell–cell adhesion molecule that has been reported to be highly expressed in advanced epithelial ovarian carcinomas (Ong et al., 2000) and failed to form xenografts in mice (Davies et al., 2003).

Therefore, in the present study we have investigated the isoform expression pattern of 3β-HSD1 and 3β-HSD2 in hOSE both *in vivo* and *in vitro* and their transcriptional regulation by the pro-inflammatory cytokine IL-1α as well as the anti-inflammatory cytokine IL-4. Moreover, we have tested whether OSE-C2 cells respond similarly to primary hOSE cells. Due to limitations in primary tissue, we have used OSE-C2 cell line to examine further the role of the apoptotic agents, TGF-β1 and RA, in the regulation of 3β-HSD mRNA levels.

2. Subjects and methods

2.1. Subjects

hOSE cells for *in vitro* experimentation or normal ovarian tissue for histological studies were collected from pre-menopausal women (mean age 36 yrs) who underwent surgery for non-malignant, benign gynaecological disorders, such as fibroids, heavy menstruation and pelvic pain. Written consent to obtain tissue was provided by all patients and the Lothian Research Ethical Committee (LREC) approved the project (project number 05/S1103/14).

2.1.1. OSE-C2 cells

The OSE-C2 cell line immortalised from a primary hOSE cell culture collected from a pre-menopausal woman, has been previously described (Davies et al., 2003). Briefly, cells were immortalised with the catalytic subunit of telomerase (hTERT) and a temperature-sensitive form of the oncogene, SV40 Large T antigen (tsT). OSE-C2 cells were maintained and tested at the permissive temperature of 33 °C (Davies et al., 2003).

2.2. Cell culture of primary hOSE cells

Ovarian surface epithelial cells were cultured as previously reported (Hillier et al., 1998; Kruk et al., 1990). Primary hOSE cells were retrieved by brushing of the ovarian surface, then placed in 75 cm^2 donor-calf serum pre-coated flasks (Corning Inc. Glass Works; Corning NY) and cultured in a humidified tissue culture incubator gassed with 95% air–5% CO_2 at 37 °C until confluence (between 2 and 4 weeks). The culture medium contained MCDB 105:M199 (1:1, v/v) culture media, supplemented with 15% (v/v) of fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 μg/mL streptomycin and 50 IU/mL penicillin (Invitrogen Life Technologies, Inc.; Renfrewshire, UK and Sigma Chemical Co.; Poole, UK). Purity of epithelial cultures was verified with phase-contrast microscopy. Cytokeratin staining (5, 6, 8 and 17) was performed in selective cases with a commercial mouse monoclonal antibody (not shown) (Dako, Ely; Cambridgeshire, UK).

2.3. Cell culture of OSE-C2 cells

OSE-C2 cells were cultured as primary hOSE cells with minor modifications. Briefly, cells were grown at 33 °C in T165 flasks (Corning) in MCDB 105:M199 culture media (1:1, v/v) containing 2 mmol/L of L-glutamine and 10% of FBS. Culture medium did not contain antibiotics. Medium was changed every 3 days and confluence was achieved in around 5 days. An epithelial phenotype of OSE-C2 cells was ascertained using a mouse monoclonal antibody that immunoreacts with cytokeratins 5, 6, 8 and 17.

2.4. Experimental treatments of primary hOSE and OSE-C2 cells

Once confluent, cells were enzymatically digested with 0.05% trypsin (w/v) and 0.5 mM EDTA (Sigma) at 37 °C for 5 min. Cell suspensions were then washed with Dulbecco's phosphate buffer saline (DPBS) (Sigma) and centrifuged twice prior to reconstitution of resultant cell pellets in 1 mL of pre-warmed culture medium. After cell viability assessment with a haemocytometer and trypan blue staining exclusion (viability ranged between 80 and 95%), 3.5×10^5 of viable cells in a total of 2 mL of culture medium were distributed in 6-well plastic culture plates (Corning). Cell attachment was allowed for 24 h before serum-starvation for another 24 h in culture medium that contained 0.01% BSA (Sigma) instead of FBS, followed by experimental treatments with serial doses of IL-1α, IL-4 (R&D systems; Abingdon Science Park, UK), RA (Sigma) and TGF-β1 (Preprotech; London, UK). In order to investigate the potential involvement of the inflammatory nuclear factor-κB (NF-κB) and mitogenic p38 MAPK signalling transduction pathways in IL-1α and IL-4 responses, 1 μM BAY117082 (Merck; Beeston, Nottingham, UK), a selective inhibitor of the IκB (an intermediate mediator of NF-κB pathways) and SB203580 (Merck), a selective inhibitor of p38 MAPK, were included in the culture media in the presence or absence of IL-1α or IL-4. Doses of inhibitors were selected per manufacturers' guidance. All other reagents were obtained from Sigma.

2.5. Immunohistochemistry

Ovarian sections from three separate ovaries that were embedded in paraffin blocks were cut with a microtome and subjected to immunostaining for 3β-HSD. The 3β-HSD polyclonal rabbit antibody was raised against human recombinant 3β-HSD2 and immunoreacts with both human isoforms (Thomas et al., 2002). Slides were deparaffinised in xylene, serial dilutions of ethanol (100–50%), PBS (Sigma) and distilled H_2O. Following permeabilisation using a microwave heating step in 0.01 M of sodium citrate buffer (Sigma) for 15 min and cooling down for another 20 min under running tap water, sequential blocking steps of endogenous peroxidase (3% H_2O_2 in H_2O (v/v); Sigma) and avidin, biotin (Vector; Peterbourough, UK) were performed. Each step was followed by PBS washes for 5 min. Slides were then submitted to a non-immunised blocking step for 20 min and overnight incubation with the 3β-HSD antibody at 4 °C. The antibody was diluted in 1:6000 in normal goat serum (NGS) (1:5 in PBS (v/v) supplemented with 5% BSA (w/v)). Negative slides incubated with mock rabbit IgG_1 antibody were run routinely at matched concentrations. Following washes with PBS supplemented with 0.05% Tween 20 (v/v) (PBST; Sigma), sequential incubations with anti-rabbit biotinylated IgG_1 (Vector) and RTU ready-to-use ABC kit (avidin–biotin complex; horse radish peroxidase (HRP)-conjugated; Vector) for 1 h each were performed. Washing steps with PBST were performed between each step for 5 min thrice. Finally, slides were submitted to staining with HRP-conjugated diaminobenzidine (DAB; Vector) chromagen for 5 min followed by haematoxylin staining and dehydration with serial doses of ethanol (50–100%) and xylene. Slides were mounted with coverslips using pertex (Sigma). Visualisation and capture of pictures was achieved with an Olympus Provis microscope (Olympus Optical, London, UK).

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Table 1
Mean dCt values of target genes in Taqman quantitative PCR

Cell type	3β-HSD1 mean dCt	3β-HSD2 mean dCt
hOSE cells	14.03	18.12
OSE-C2 cell line	20	28.2

Mean dCt reflects Ct of target gene relative to Ct of 18S as the internal reference.

2.6. RNA extraction and quantity measurement

Following *in vitro* treatments, cells were harvested and homogenised in lysis buffer (RLT buffer, Qiagen, West Sussex, UK) before RNA extraction with the RNeasy mini-kit (Qiagen) per manufacturer's guidance. Exclusion of genomic DNA was achieved with DNase treatment of samples, on-column, with the RNase free DNase set (Qiagen) according to supplier's protocol. Purification and quantification were assessed using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies Inc, Wilmington, DE, USA).

2.7. Quantitative measurement of 3β-HSD1 and 3β-HSD2 mRNA

Quantitative Taqman Real-Time PCR was performed to measure relative expression levels of 3β-HSD1 and 3β-HSD2 mRNA in response to treatments. Briefly, pure RNA (200 ng) was reversed transcribed to cDNA using the RT-Reagent kit (Applied Biosystems, Applera, Warrington, UK) per supplier's instructions in a final reaction of 10 μL. Then, Real-Time PCR was performed using commercial Applied Biosystems reagents. Briefly, 2 μL cDNA was used as a template mixed with 1× universal Taqman master cocktail (Applied Biosystems) and the specific set of 1× primer/probe mixture. Both 3β-HSD1 and 3β-HSD2 primer/probe sets were purchased pre-validated (Assay-on-Demand Systems, Applied Biosystems). A ribosomal 18S primer/probe set was also included and served as an internal reference control. Mean values reflecting the PCR cycle when the target transcript started to be accumulated relative to 18S (mean dCt in a 40 cycle PCR reaction) are illustrated in Table 1. Values more than 36 out of 40 PCR cycles were assessed as beyond the limit of robust detection; however they were included in analysis for comparison reasons. Each reaction was carried out in duplicate. Samples were evaluated in 96-well plates using an ABI Prism 7900 Sequence Detector (Applied Biosystems).

2.8. Statistical analysis

Data sets are presented as means and standard errors of at least three independent sets of replicates. Basic statistical analysis was run using the GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, USA). Multiple comparisons were performed with one-way ANOVA and Neuman–Keuls post hoc tests, whilst single comparisons were achieved with paired Student's *t*-tests. Statistical significant were considered at p-values ≤ 0.05. All values given in Section 3 and figure legends reflect statistical difference relative to untreated control cells, unless otherwise specified.

3. Results

3.1. Expression of 3β-HSD protein in the human ovarian surface epithelium

Staining with the 3β-HSD antibody in a representative ovary is illustrated in Fig. 1. 3β-HSD protein was immunolocalised in the ovarian surface epithelium of the pre-menopausal human ovary as revealed with immunohistochemistry with a polyclonal rabbit 3β-HSD antibody (block arrow, Fig. 1A). Intriguingly, expression levels varied among different cells of the ovarian surface. Specificity of the antibody was tested by using an antral follicle as positive control. As illustrated in Fig. 1B, follicular cells and particularly membrana granulosa and theca interna were immunostained for 3β-HSD (block arrow). Incubation with unconjugated IgG_1 rabbit antibody was used for negative control (insets in Fig. 1A and B).

3.2. Expression pattern of 3β-HSD1 and 3β-HSD2 mRNA in primary hOSE cells and OSE-C2 cell line

Because the 3β-HSD antibody used in immunolocalisation studies reflected total 3β-HSD protein (3β-HSD1 and/or 3β-HSD2 proteins), relative isoform expression was investigated with quantitative Real-Time PCR using isoform-specific primer/probe sets. Primary hOSE cells obtained from 7 individual patients were positive for both 3β-HSD1 and 3β-HSD2 mRNA with a mean 12-fold increase of 3β-HSD1 mRNA as opposed to 3β-HSD2 mRNA ($n = 7$, $b = p < 0.05$; Fig. 2, upper panel). On the other hand OSE-C2 cells displayed only 3β-HSD1 mRNA, 3β-HSD2 mRNA being immeasurable ($n = 3$, $c = p < 0.001$; Fig. 2B, lower panel). Raw dCt values are listed in Table 1.

3.3. Regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-1α in primary hOSE cells and OSE-C2 cell line

Stimulation of primary hOSE cells with IL-1α in a dose–response manner for 48 h as a proxy to mimic ovulation-associated inflammation resulted in a 3-fold suppression of 3β-HSD1 mRNA as assessed by quantitative Real-Time PCR (Taqman). This effect was dose-dependent (0.02 and 0.5 ng/mL) with a maximal down-regulation when IL-1α (0.5 ng/mL) was added ($n = 4$, $b = p < 0.05$; Fig. 3, upper panel). At the same time, 3β-HSD2 mRNA was up-regulated in response to IL-1α in a dose-dependent manner. The maximal fold-change (4-fold-change) was observed with 0.1 and 0.5 ng/mL of IL-1α ($n = 4$, $b = p < 0.05$, $c = p < 0.01$; Fig. 3, lower panel).

In OSE-C2 cells, no effect on 3β-HSD1 mRNA by IL-1α was demonstrable, probably due to very low basal expression levels (data not shown). Moreover, loss of 3β-HSD2 mRNA was not restored by IL-1α treatment (data not shown).

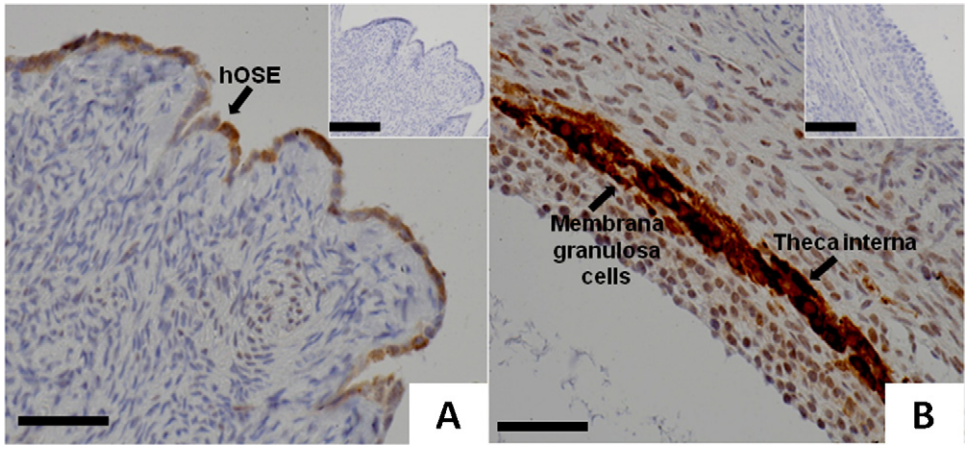

Fig. 1. Expression of 3β-HSD in hOSE. (A) Immunolocalisation of 3β-HSD in the hOSE layer of a representative human ovary. Disparity of staining intensity among different cells of the ovarian cell surface is noticed. The inset represents a negative control. (B) Immunostaining of an antral follicle for 3β-HSD. Immunopositivity is observed in membrana granulosa and theca interna. The inset reflects a negative slide. Scale bars represent 50 μm.

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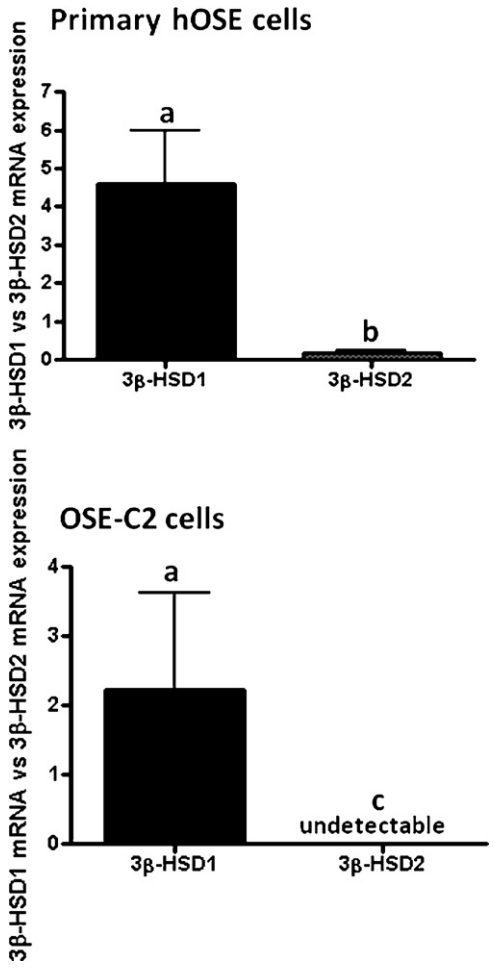

Fig. 2. The pattern of mRNA expression of 3β-HSD isoforms in primary hOSE and OSE-C2 cells. Upper panel: The hOSE cells were separately collected from seven patients ($n = 7$, $b = p < 0.05$; upper panel). Lower panel: In OSE-C2 cells, only 3β-HSD1 mRNA was detectable ($n = 3$, $c = p < 0.001$; lower panel).

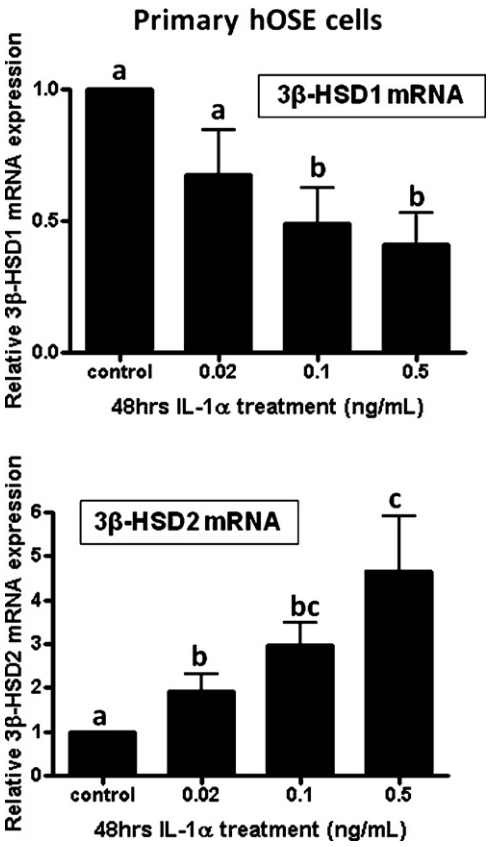

Fig. 3. The effect of IL-1α on 3β-HSD mRNA levels in primary hOSE cells. Combined data of four independent replicates of primary hOSE cells treated with the indicated doses of IL-1α for 48 h. Upper panel: 3β-HSD1 ($b = p < 0.05$); lower panel: 3β-HSD2 ($b = p < 0.05$, $c = p < 0.01$).

3.4. Regulation of 3β-HSD1 and 3β-HSD2 mRNA by IL-4 in primary hOSE cells and OSE-C2 cell line

Following IL-1α treatment, primary hOSE and OSE-C2 cells were treated with the 'anti-inflammatory' cytokine IL-4 for 48 h and quantification of 3β-HSD1 and 3β-HSD2 mRNA levels was performed. In primary hOSE cells obtained from 4 separate patients, IL-4 had a substantial stimulatory dose-dependent effect on both 3β-HSD1 and 3β-HSD2 mRNAs (15-fold-change and 70-fold increase, respectively, $n = 4$, $b = p < 0.05$, $c = p < 0.001$ Fig. 4A). Treatment of OSE-C2 cells with IL-4 in a time- and dose-dependent manner resulted in a substantial increase of 3β-HSD1 mRNA ($n = 3$, $b = p < 0.001$, Fig. 4B, upper panel). Combined data from 3 independent replicates showed that 3β-HSD2 mRNA levels did not alter with extended IL-4 treatment times (data not shown). In contrast, 8 h treatment with serial doses of IL-4 dose-dependently increased 3β-HSD1 mRNA with a maximal effect when 0.25 ng/mL of IL-4 was added ($n = 3$, $b = p < 0.05$, c, $d = p < 0.001$, Fig. 4B, lower panel).

3.5. Involvement of NF-κB inflammatory signalling pathway in IL-1α- and IL-4-mediated 3β-HSD mRNAs in primary hOSE cells

In order to investigate if the inflammatory pathway NF-κB was involved in the mechanism by which cytokines regulate transcription of 3β-HSD1 and 3β-HSD2 mRNA, we treated primary hOSE cells with 1 μM BAY117082, a selective inhibitor of IκB, in the presence or absence of 0.5 ng/mL of IL-1α or 0.5 ng/mL of IL-4 for 48 h (Fig. 5). BAY117082 reversed IL-1α-suppressed 3β-HSD1 mRNA levels, whereas it did not affect the IL-4 response ($n = 4$, $b = p < 0.05$, $c = p < 0.001$; Fig. 5, upper panel). Regarding 3β-HSD2 mRNA regulation, the IL-1α effect was also blocked with no effect with respect to IL-4 stimulation (Fig. 5, lower panel).

3.6. Involvement of p38 MAPK signalling pathway in IL-1α- and IL-4-mediated 3β-HSD mRNA expression in primary hOSE cells

We also addressed whether the p38 MAPK signalling pathway participated in the transduction of 3β-HSD mRNA transcriptional regulation. Primary hOSE cells were treated with 10 μM SB203580, a selective pathway inhibitor. Intriguingly, this inhibitor alone down-regulated 3β-HSD1 mRNA levels, although it did not further affect responses observed with cytokines alone ($n = 3$, $b = p < 0.05$; Fig. 6, upper panel). On the other hand, stimulatory effects of 0.5 ng/mL IL-1α and IL-4 on 3β-HSD2 mRNA levels were completely blocked in the presence of the inhibitor ($n = 3$, $b = p < 0.05$, $c = p < 0.001$; Fig. 6, lower panel).

3.7. Regulation of 3β-HSD1 mRNA by TGF-β1 and all-trans retinoic acid (RA) using OSE-C2 cell line as a model of hOSE

Because of restricted primary tissue availability and given that OSE-C2 cells respond to cytokines similarly to primary hOSE cells, we further investigated progesterone pre-receptor metabolism

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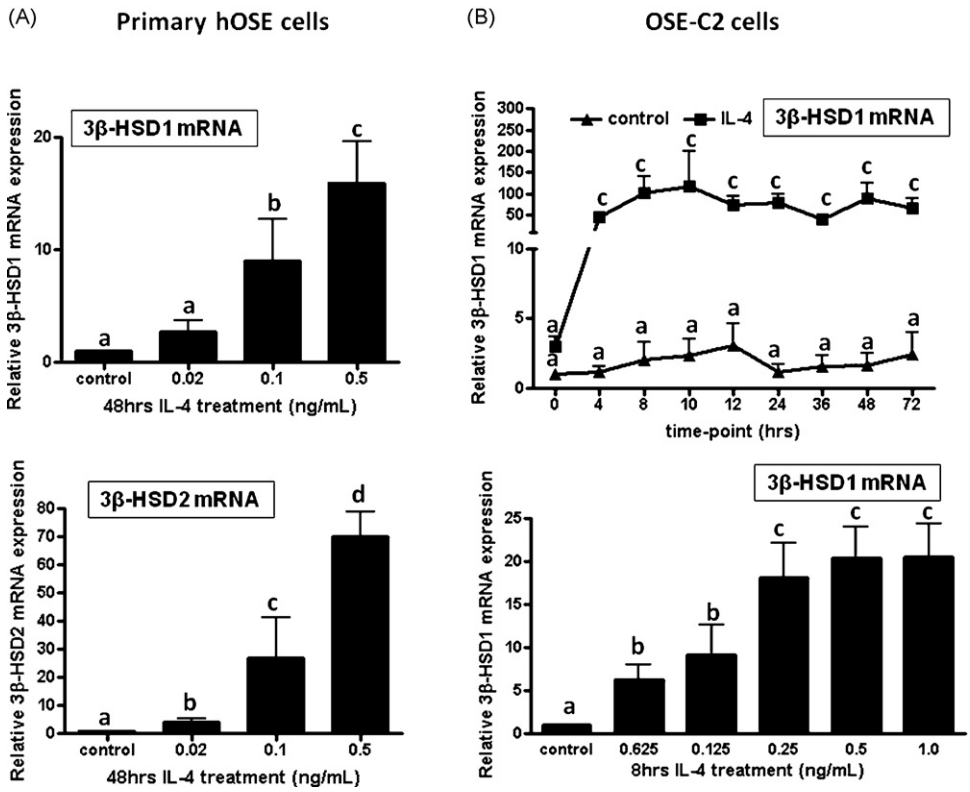

Fig. 4. The effect of IL-4 on 3β-HSD mRNA levels in primary hOSE and OSE-C2 cells. (A) Combined data of four independent experiments of primary hOSE cells treated with various doses of IL-4 for 48 h—3β-HSD1 (upper panel) and 3β-HSD2 (lower panel) mRNA ($b = p < 0.05$; c, $d = p < 0.001$). (B) Combined data of three independent experiments of 3β-HSD1 mRNA levels in OSE-C2 cells treated with IL-4 (0.5 ng/mL) for the indicated times (upper panel; $c = p < 0.05$) or treated with various doses of IL-4 for 8 h ($b < 0.05$, $c = 0.001$, lower panel).

using this cell line as a model. OSE-C2 cells were treated with apoptotic factors RA and TGF-β1 at various different doses and time-points (Fig. 7). Then, 3β-HSD1 mRNA expression levels in response to these factors were measured. Treatment with RA resulted in a dose-dependent stimulation of 3β-HSD1 mRNA with an average 18-fold increase obtained with 1 μM RA treatment for 48 h. This effect was not significantly altered at the two different time-points tested ($n = 4$, $b = p < 0.05$, $c = p < 0.01$; Fig. 7, upper panel). Culture of OSE-C2 cells in the presence of TGFβ-1 resulted in induction of 3β-HSD1 mRNA at both doses tested. Notably, the effect was maximal at the 8 h time-point (35-fold-change) with moderate but significant reduction after 48 h of treatment ($n = 4$, $b = p < 0.05$, $c = p < 0.01$; Fig. 7, lower panel).

We next tested if there was a synergistic effect between these two components on 3β-HSD1 mRNA levels. Remarkably, the combination of both RA and TGF-β1 for 8 h and 48 h in four independent replicates increased 3β-HSD1 mRNA expression levels at least 100-fold ($n = 4$, $b = p < 0.05$, $c = p < 0.01$, $d = p < 0.001$; Fig. 8).

4. Discussion

Herein, we demonstrate both 3β-HSD1 and 3β-HSD2 mRNA functionality in the ovarian cell surface. For the first time, we show multiple mechanisms of pre-receptor progesterone intracrine formation at the ovarian cell surface with profound implications in the development of novel pharmaceutical strategies for ovarian disorders such as EOC. We confirmed previous reports for abrogation of 3β-HSD1 mRNA by IL-1α (Rae et al., 2004b). We also ascertained expression of 3β-HSD2 mRNA that was remarkably induced in response to IL-1α treatment as well as stimulation of both isoforms by the anti-inflammatory IL-4. We demonstrated that the p38 MAPK signalling pathway may play a major role in the anti-inflammatory responses of IL-1α and IL-4 and also that a NF-κB pathway is involved in inflammatory-mediated changes that IL-1α exerts in the transcriptional regulation of 3β-HSD1 and 3β-HSD2. Also novel is the finding that 3β-HSD1 mRNA levels in OSE-C2 cells are profoundly induced by the apoptotic and anti-proliferative factors TGF-β1 and RA, with an even more substantial transcript accumulation when both agents are combined.

Localisation of 3β-HSD in the human ovarian surface epithelium is suggestive of a capacity of this ovarian compartment to control local bioavailability of progesterone *in situ* for binding to the cognate progesterone receptor (PR). Remarkably, we noticed differences in the intensity of 3β-HSD staining among different cells of the surface suggestive of disparity in steroidogenic profile within the same compartment. Attenuated 3β-HSD expression might well represent a defining feature of (pre)-neoplastic transformation and this demands further investigation. In support of this, the presence of steroidogenic acute regulatory (StAR) protein, 3β-HSD and PR has previously been demonstrated in ovarian cancers, all involved in progesterone formation and action, and linked with a good prognosis of the disease (Abd-Elaziz et al., 2005).

Because the 3β-HSD antibody reacts with both isoforms, we further assessed the isoform expression pattern with quantitative Real-Time PCR using isoform-specific primer/probe sets. To assess this, we used primary hOSE cell cultures as well as the immortalised cell line, OSE-C2. Notably in primary hOSE cells both transcripts were present with 3β-HSD1 mRNA expressed at higher levels compared to 3β-HSD2 mRNA. This predominance was further confirmed in OSE-C2 cells, where 3β-HSD2 mRNA was not detectable. This expression pattern is different from what has been reported for the rest of the ovary where 3β-HSD2 predominates,

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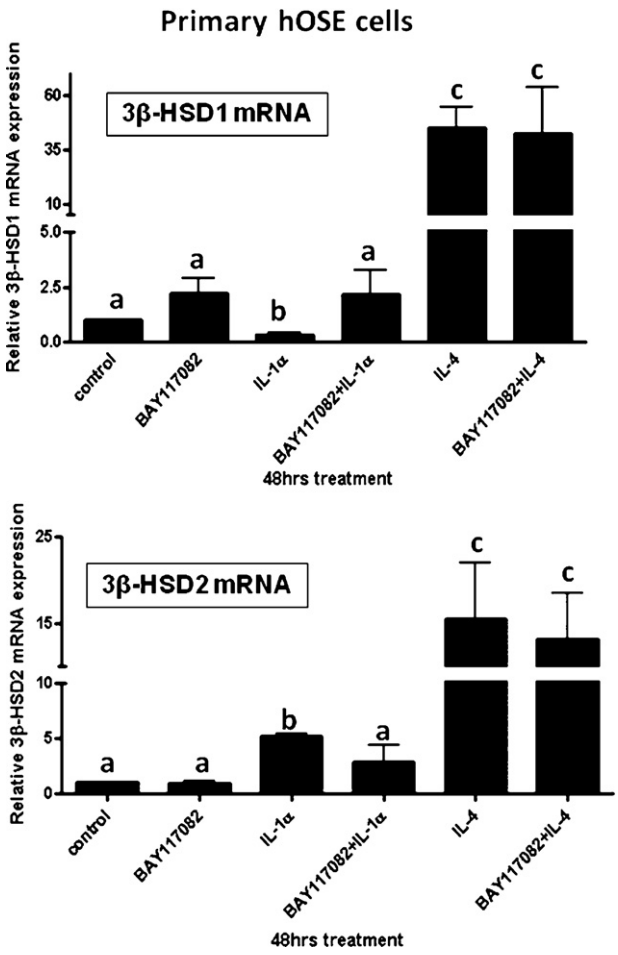

Fig. 5. The effect of BAY117082, a selective NF-κB pathway inhibitor, on cytokine transduction of 3β-HSD mRNAs in primary hOSE cells. hOSE cells from four independent patients were variously treated with IL-1α (0.5 ng/mL), IL-4 (0.5 ng/mL) and/or BAY117082 (1 μM) for 48 h prior to isolation of RNA for qRT-PCR analysis (upper panel) 3β-HSD1 mRNA and (lower panel) 3β-HSD2 mRNA ($b = p < 0.05$, $c = p < 0.001$).

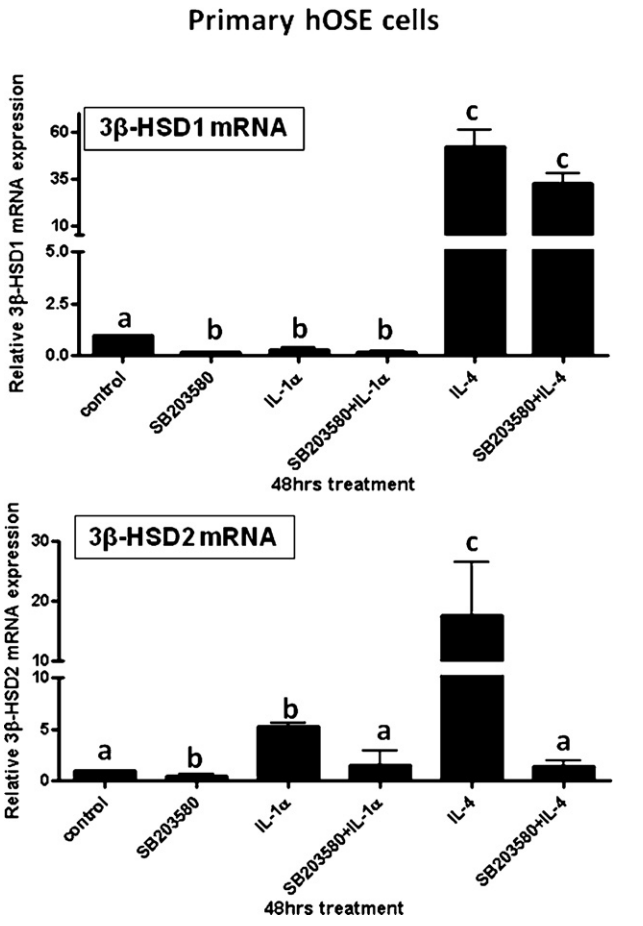

Fig. 6. The effect of SB203580, a selective p38 MAPK kinase pathway inhibitor, on transduction of 3β-HSD1 and 3β-HSD2 mRNAs by IL-1α and IL-4 in primary hOSE cells. Cells from three separate patients were treated with IL-1α (0.5 ng/mL), IL-4 (0.5 ng/mL) and/or SB203580 (10 μM) for 48 h, after which RNA was isolated and qRT-PCR performed (upper panel: $n = 3$, $b = p < 0.05$, $c = p < 0.001$; lower panel: $b = p < 0.05$, $c = p < 0.001$).

with emergence of low levels of 3β-HSD1 only in the corpus luteum (Rheaume et al., 1991). The relevance of 3β-HSD1 preponderance in this compartment could be related to the low capacity of these cells to produce *de novo* steroids from cholesterol at least *in vitro* (Rae et al., 2004b; Rembiszewska and Brynczak, 1985).

As previously demonstrated, IL-1α treatment of primary hOSE cells had an inhibitory effect on 3β-HSD1 mRNA expression, implicating that, physiologically at the peri-ovulatory period, the steroidogenic capacity of hOSE is minimised and this may probably be a key event for the initiation of ovulation (Rae et al., 2004b). On the other hand, concomitant IL-1α-induced 3β-HSD2 mRNA expression indicates an efficient mechanism of hOSE cells to maintain basic steroid formation, at least of progesterone. Another mechanism to restore intracrine formation in hOSE is via IL-4 treatment. IL-4 *in vivo* is secreted by peripheral mononuclear blood cells that infiltrate the ovary and starts to accumulate at the peri-ovulatory period with peak production at the luteal phase of the menstrual cycle when progesterone secretion is also at its peak (Hashii et al., 1998). Collectively, progesterone accumulation and downstream signalling peri-/post-ovulatory highlight an efficient mechanism of hOSE to minimise genetic instability and protect clonal expansion of any transformed cell that could lead to EOC (Murdoch and Van Kirk, 2002). Fundamentally, progesterone has been proven anti-inflammatory, anti-proliferative and apoptotic (Bu et al., 1997; Ivarsson et al., 2001; Rae et al., 2004a). Progesterone triggers apoptosis to attenuate potential genetic damage of the post-ovulatory ovarian surface. In sheep, progesterone treatment resulted in a decline of 8-oxoguanine adducts through induction of polymerase-β and poly(ADP-ribose) polymerase, both involved in DNA repair. Moreover, reports suggest that progesterone exerts its apoptotic and anti-proliferative effects through up-regulation of the tumour suppressor p53 protein (Murdoch and Van Kirk, 2002b). The latter has been also seen in human ovarian cancer cell lines, where progesterone exerted anti-proliferative and apoptotic effects through arrest of cells at G1 phase of the cell cycle and concomitant stimulation of the p53 mRNA (Bu et al., 1997). Also, progesterone has been demonstrated to have a role in resolution of ovulation-associated inflammation as it reversed IL-1α-induced inflammatory cyclooxygenase-2 (COX-2) mRNA (Rae et al., 2004a).

We were unable to demonstrate an IL-1α pro-inflammatory effect on 3β-HSD1 mRNA levels in OSE-C2 cells. This limitation was probably due to the fact that basal 3β-HSD1 mRNA levels in this cell type were already much lower than those of hOSE cells (data not shown) and therefore it was not feasible practically to demonstrate down-regulation of the transcript in response to this proxy. Also, absence of 3β-HSD2 mRNA was not reversed with IL-1α treatment (data not shown). Collectively, the attenuation of 3β-HSD1 and 3β-HSD2 mRNA could be a defining feature of (pre)-neoplastic transformation and this will be the subject of further investiga-

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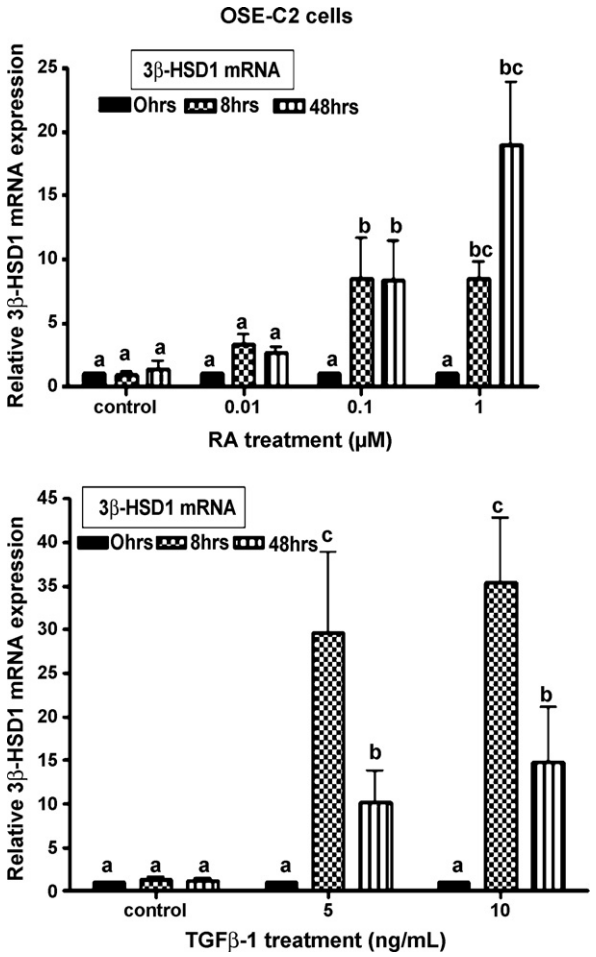

Fig. 7. The effects of all-trans retinoic acid (RA) and TGF-β1 on 3β-HSD1 mRNA levels of OSE-C2 cells. Upper panel: Four independent cultures of OSE-C2 cells were treated with serial doses of RA (1 μM) for 8 and 48 h ($n = 4$, $b = p < 0.05$, $c = p < 0.01$). Lower panel: Four separate cultures of OSE-C2 cells treated with TGF-β1 (2 doses) for 8 and 48 h ($n = 4$, $b = p < 0.05$, $c = p < 0.01$, lower panel).

tion using primary cancer cells (Papacleovoulou et al., unpublished data). On the other hand, IL-4 substantially up-regulated 3β-HSD1 mRNA, consistent with the IL-4 effect on primary hOSE cells, establishing this cell line as an appropriate model to study

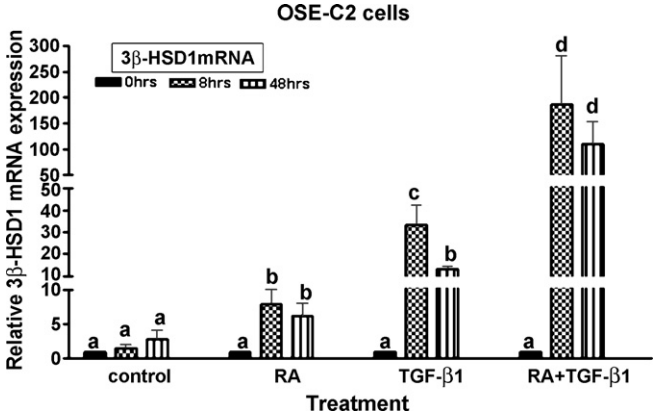

Fig. 8. Synergistic effect of all-trans-retinoic acid (RA) and TGF-β1 on 3β-HSD1 mRNA transcript levels in OSE-C2 cells. In four independent experiments, OSE-C2 cells were treated for 8 or 48 h with RA (1 μM) or TGF-β1 (5 ng/mL) or in combination ($n = 4$, $b = p < 0.05$, $c = p < 0.01$, $d = p < 0.001$) prior to isolation of RNA and qRT-PCR for 3β-HSD1 mRNA transcripts.

physiology and pre-neoplastic transformation of the ovarian cell surface.

Identification of mechanisms that drive IL-1α and IL-4 pro-inflammatory and anti-inflammatory responses to regulation of 3β-HSD1 and 3β-HSD2 could be potentially proved essential for prevention or treatment of ovarian disorders. We are the first to show that a pro-inflammatory NF-κB pathway is involved in transduction of 3β-HSD1 and 3β-HSD2 mRNA by cytokines at least in hOSE cells as revealed by blockage of IL-1α-mediated 3β-HSD mRNAs after treatment with a selective pathway inhibitor. Previously, it has been proposed that NF-κB and PR mutually suppress each other's activity (van der Burg and van der Saag, 1996). Precisely, it has been demonstrated that progesterone's immunosuppressive and anti-inflammatory action is subject to its ability to harness the initiation of inflammatory processes such as ovulation through inhibition of NF-κB signalling. Vice versa, NF-κB activation abrogates PR-associated action (Kalkhoven et al., 1996). Our data suggest that these effects might well be mediated by 3β-HSD regulation that essentially controls local bioavailability of the active ligand and access to PR, a crucial event for trans-activation of downstream progesterone signalling. Remarkably, the p38 MAPK pathway appears to have a role in that, since its abrogation suppressed basal 3β-HSD1 and 3β-HSD2 mRNA levels, establishing the anti-inflammatory nature of this gene. Also, inhibition of p38 MAPK action completely blocked anti-inflammatory responses of IL-4 and IL-1α with regard to 3β-HSD2 mRNA, attesting a role of this transduction signalling pathway in anti-inflammatory steroid mechanisms within hOSE. It remains to be established if IL-4 exerts its anti-inflammatory action through inhibition of NF-κB-associated pro-inflammatory responses and if concurrent activation of the p38 MAPK signalling transduction pathway is a pre-requisite for this effect.

Because of a very restricted availability of primary tissue, we used OSE-C2 cells to demonstrate that retinoic acid can enhance 3β-HSD1 mRNA, indicating that a mechanism through which it may exert its chemopreventive and pro-apoptotic effects is by regulation of 3β-HSD1 and thereby promotion of progesterone formation. Retinoic acid and its derivatives have been considered as therapeutic targets of several cancers such as leukaemia, breast cancer, cervical cancer and pancreatic adenocarcinoma (Altucci and Gronemeyer, 2001; Behbakht et al., 1996; El-Metwally et al., 2006). In ovarian cancer, retinoids have been shown to increase the survival time of mice treated with a human ovarian cancer cell line xenograft and also enhanced anti-tumour activity of cispaltin in two human EOC cell lines (Formelli and Cleris, 1993). Additionally, in human ovarian cancer cell lines, retinoid treatment resulted in increased apoptosis (Bu et al., 1997).

We also established that TGF-β1 can markedly promote intracrine formation of progesterone. Because we used OSE-C2 cells to test that assumption, only data regarding the major 3β-HSD transcript (3β-HSD1) is available. Once again, these data suggest that pro-apoptotic and anti-proliferative effects of TGF-β1 might well be exerted through enhancement of progesterone pre-receptor signalling. It has been previously demonstrated that TGF-β1 supplementation in pre-neoplastic and tumour ovarian cells attenuated cell proliferation, promoted DNA fragmentation and reduced anti-apoptotic bcl-2 protein (Berchuck et al., 1992; Choi et al., 2001). Intriguingly, it has been also shown that TGF-β1 up-regulated 3β-HSD2 activity in human lutein-granulosa cells and 3β-HSD2 mRNA in human adrenal cells (Lebrethon et al., 1994; McAllister et al., 1994). It is therefore interesting to investigate if this is reproducible in 3β-HSD2 of hOSE cells.

Finally, we treated OSE-C2 cells with TGF-β1 in the presence of retinoic acid. Expectedly, up-regulation of 3β-HSD1 mRNA was noticed; however 3β-HSD1 mRNA stimulation was far higher

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than that achieved with RA or TGF-β1 alone, implying a synergistic action of these two agents. A proposed mechanism for anti-proliferative action of RA in human pancreatic cancer and rat prostatic epithelial cells is through induction of TGF-β1 secretion (Danielpour, 1996; Singh et al., 2007). As such, co-treatment of both factors in OSE-C2 cells could result in a parallel autocrine and paracrine signalling of TGF-β1 thereby driving progesterone formation. Furthermore, in mice, it has been demonstrated that retinoic acid participates in TGF-β1 differential immune-associated signalling by promoting TGF-β1 anti-inflammatory responses (Mucida et al., 2007).

In summary, we have presented major immune and growth factors that are involved in enhancement of pre-receptor progesterone metabolism with profound implications in the development of therapeutic strategies for ovarian disorders and more importantly EOC. We have elucidated that p38 MAPK signalling pathway has an indispensable role at this process. Moreover, combination of TGF-β1 with retinoic acid could be proved beneficial for driving progesterone signalling in the human ovary.

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